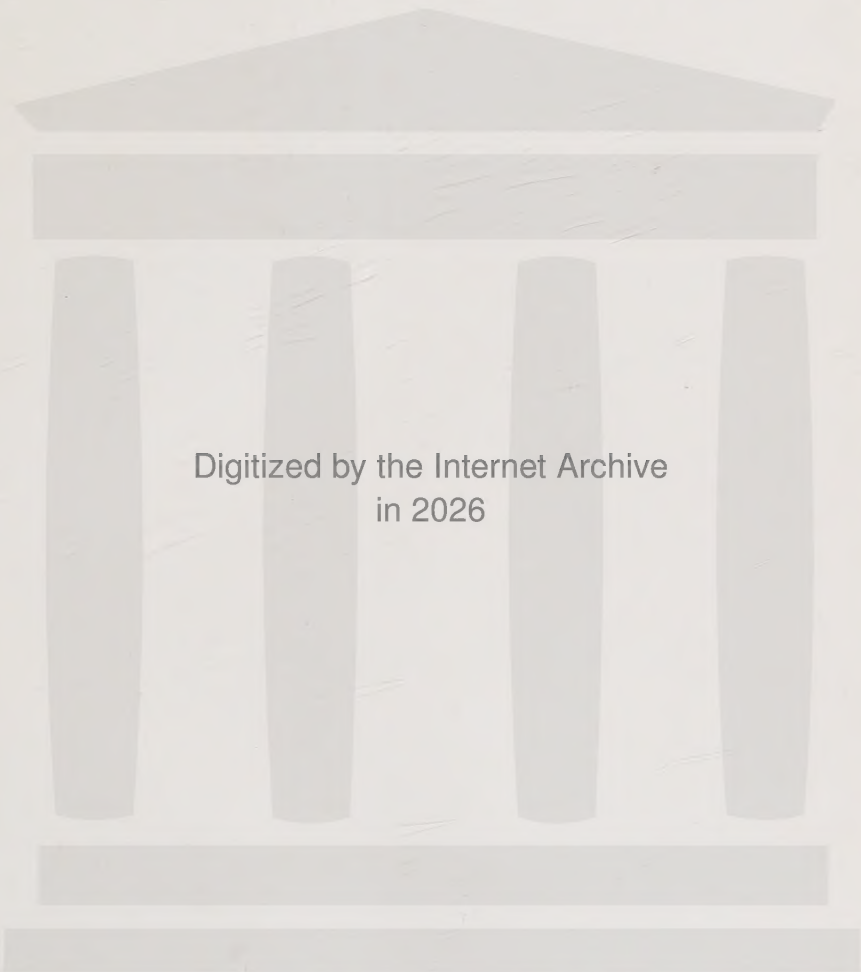


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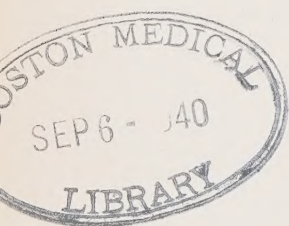
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VOLUME 77

MAY, JUNE, JULY, AUGUST, 1940

THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA, PA.

30117



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SUPEROVULATION IN RABBITS¹

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The desirability of obtaining large numbers of rabbit ova for physiological studies of the ovum has led to an investigation of the usefulness of several gonadotropic materials for this purpose. The objectives were to ripen as large a number of follicles as possible and to determine whether the ova obtained at ovulation were fertilizable. Stimulation of follicles is readily obtained in rabbits by the injection of the gonadotropic material from mare's serum or of pituitary gonadotropic extracts. Accordingly we have tested three preparations each of mare's serum and pituitary extracts. To determine whether the ova are fertilizable a number of the animals used were mated at the time of the administration of an ovulating injection.

The standard procedure employed was as follows:

Four to 6 month old rabbit does of medium-sized strains were injected subcutaneously six times at 12 hour intervals with a priming dose of the gonadotropic material. At 8 to 12 hours after the last priming injection an intravenous injection was made to induce ovulation. Varying priming and ovulating dosages were employed in an attempt to ascertain the optimum dosage (see table 1).

We have taken as our standard of comparison a course of injections of FSH and FSH plus LH (ovulating dose) obtained from sheep pituitary, previously ascertained to be optimum (Pincus, '39). The FSH powder was dissolved in water so that 12 mg. constituted the total priming dose and

¹Aided by a grant from the Buffington Company.

TABLE 1

The effects of various priming and ovulating doses of mare's serum and pituitary extracts upon ovulation in the rabbit

COLUMN NO.	PRIMING MATERIAL INJECTED	PRIMING DOSE	OVULATING DOSE	NUMBER OF RABBITS	AVERAGE NUMBER OF EGGS OVULATED	RANGE IN EGG NUMBER	REMARKS
1	Antostab	100 m.u.	120 m.u. Antostab	2	11.5	10-13	Cystic follicles
2	"	" "	2 mgm. LH	2	12.5	3-22	Mated
3	"	60 m.u.	15 mgm. FSH	2	0.0	0	Cystic ovaries
4	"	90 m.u.	2 mgm. LH None	2	3.0	0-6	Mated
5	"	" "	15 mgm. FSH	2	9.0	6-12	Cystic ovaries
6	"	130 m.u.	2 mgm. LH " "	3	18.3	10-30	Cysts and blood follicles in ovaries
7	"	150 m.u.	" "	1	7.0	7	Cystic ovaries
8	"	" "	" "	2	13.2	12-15	Mated, cystic ovaries
9	"	160 m.u.	" "	2	8.5	7-10	Cystic ovaries
10	PMS	72 m.u.	15 mgm. FSH	4	8.0	6-9	Cystic ovaries
11	"	144 m.u.	2 mgm. LH	2	0.0	0	Cystic follicles
12	"	" "	" "	2	4.5	2-7	Mated, cystic follicles, No fertilization
13	"	72 m.u.	72 m.u. PMS	3	11.0	8-16	Cystic follicles
14	"	108 m.u.	2 mgm. LH 10 m.u. PMS	1	0.0	0	Cystic follicles
15	"	"	2 mgm. LH 7 m.u. PMS	1	6.0	6	Cystic follicles
16	Anteron	198 r.u.	2 mgm. LH 33 r.u. Anteron	1	0.0	0	Large cysts and blood follicles
17	"	19.8 r.u.	16.5 r.u. Anteron	2	0.0	0	Many large cystic and blood follicles
18	"	19.8 r.u.	19.8 r.u. Anteron	1	11.0	11	Mated, cystic and blood follicles
19	"	9.9 r.u.	8.2 r.u. Anteron	2	28.0	24-32	Mated, cystic and blood follicles; no fertilization
20	"	"	None	1	0.0	0	Cystic and blood follicles

				17	31.9	7-82	Mated; few or no cystic or blood follicles
23							
24				2	27.0	24-30	
25				1	17.0	17	Mated
26				1	24.0	24	
27				4	21.5	15-30	
28				4	29.0	14-70	
29	Horse AP1	4.8 mgm.		3	29.7	26-32	
30				2	16.0	16	Mated
31				2	22.0	5-39	
32				2	32.0	20-44	Mated
33				1	30.0	30	
34				1	0.0	0	
35	AP2	1.5 mgm.		2	26.0	12-40	Cystic follicles
36				1	0	0	Some cystic follicles
37				2	53.0	39-76	
38				1	13.0	13	
39				3	24.0	11-47	
40				2	30.0	8-52	Mated
41				2	26.0	24-48	
42				2	38.5	18-59	
43				2	26.5	21-32	
44				3	32.3	36-38	
45				1	9.0	9	
46				1	43.0	43	Mated
47				2	47.0	43-51	
48				1	18.0	18	Mated adult
49				1	33.0	33	
50				1	44.0	44	Mated
51				2	63.5	52-75	Mated
52				5	47.8	22-76	
53				2	73.0	64-82	Mated
54				2	35.5	22-49	Mated
55				3	39.0	35-41	
56				2	62.0	48-76	
57				2	50.0	33-67	Mated

15 mg. FSH plus 2 mg. LH constituted the ovulating dose. The rabbits were ordinarily sacrificed at 16 to 22 hours after the ovulating injection, the eggs flushed from the fallopian tubes and counted. When an accurate count could not be made the ovulation points on the ovaries were counted.

The three extracts of pregnant mares' serum are Antostab, an acetone precipitate (PMS), and Anteron. The pituitary extracts are FSH and LH prepared as described by Fevold ('37). An acetone precipitate of horse pituitary (AP1) and a second pituitary extract not separated into FSH and LH fractions (AP2)².

The detailed data for various dosages and combinations of the materials used are presented in table 1. They indicate: (1) that copulation cannot substitute for the ovulating injection (columns 4 and 20); (2) that mating at the time of the ovulating injection does not especially enhance the number of ova ovulated (compare columns 1 and 2, 7 and 8, 11 and 12, 17 and 18, 22 and 23, 24 and 25, 29 and 30, 31 and 32, 39 and 40, 45 and 46, 49 and 50, 52 and 53, 54 and 55, 56 and 57); (3) that substituting FSH for the mare's serum extracts Antostab and PMS in the ovulating injection does not increase the number of eggs ovulated (columns 1 to 15); (4) that substituting mare's serum extract for FSH in the ovulating injection gives a good ovulation but less, on the average, than with FSH itself (columns 22 to 27); (5) that after a given priming with a crude horse pituitary preparation an ovulating injection of the crude preparation alone or with LH added is as effective as FSH plus LH (columns 28 to 32); (6) that above a lower limit increasing the ovulating dosage of a crude pituitary extract does not increase the number of eggs ovulated (columns 36 to 48).

Comparison of the extracts of mare's serum with the pituitary extracts indicates that the former are less efficient in superovulating. This is best shown by the data of table 2

² The Antostab was donated by the Boots Drug Company, the Anteron by Dr. Erwin Schwenk of the Schering Company, the PMS and AP1 by Dr. A. S. Parkes of the National Institute of Medical Research, London.

wherein, omitting the data on non-ovulating animals, the number of ova ovulated are totalled. The averages are drawn from data on mated and unmated animals combined since the data of table 1 do not indicate any large difference between these. A statistical comparison of the data indicates that the pituitary extracts give a significantly higher number of eggs ovulated.

The difference between the mare's serum and pituitary extracts seems to be accountable to a qualitative difference in effect on the ovaries. The former, above a lower limiting priming dose (columns 10, 13, 21) tends to produce cystic follicles and blood follicles that fail to release ova (columns 1, 6, 7, 8, 9, 11, 14, 15, 16, 17, 18, 19, 20). If, for one reason or

TABLE 2

The average number of ova in ovulating females produced by various gonadotropic preparations

MATERIAL	NUMBER OVULATING	AVERAGE NUMBER OF EGGS PER FEMALE	RANGE IN EGG NUMBER
Autostab	15	11.8	3-30
PMS	10	8.0	2-16
Anteron	5	16.0	6-32
Sheep FSH	73	28.5	11-80
Horse AP1	14	26.8	5-70
AP2	44	39.7	8-82

another, the ovary fails to respond to the ovulating stimulus at a low priming dose, continued follicle growth results also in cystic ovaries (columns 3, 4). Again this peculiar effect of mare's serum extracts is illustrated by the short dosage range over which any ovulation may be obtained. Thus with AP2 superovulation is had with every priming dose used (from 1.5 mg. to 15.0 mg.) i.e., a tenfold dosage range whereas the lowest dosage of Anteron used (column 21) gives a low number of ova ovulated (without, be it noted, any marked follicle stimulation) and tripling this dose (columns 17 and 18) results in heavily cystic follicles with no or slight ovulation (compare also columns 1, 2, 4, 5 with 6 to 9; and 10, 13 with 11, 12, 14 and 15). Superovulation, then, may be

had with the use of mare's serum extracts, but only over a limited dosage range and rather variably. The maximum number obtained with any mare's serum preparation is 32 whereas the pituitary extracts frequently produce over 60. Cystic and blood follicles occur occasionally in the FSH and AP series but never to the extent seen in the mare's serum series.

According to Gregory ('32) rabbits of strains of the size used in these experiments produce on the average some 9 ova at an ovulation with a maximum possible number of 15. The sheep FSH and LH and the AP1 preparations averaged three times the normal and the AP2 preparation over four times. In the AP2 series it is obvious that the number of ova ovulated is roughly proportional to the priming dosage employed. The mean number of ova ovulated by AP2 is greater than in the FSH and AP1 series and the difference is statistically significant between the FSH and AP2 data. This does not necessarily mean that an unfractionated extract is superior to the fractionated one since the dosages of FSH and FSH plus LH employed may not have been balanced to give maximum ovulation (see Foster and Fevold, '35; and Fevold, '37). If, however, this difference were genuine, then the superiority of an unfractionated extract to the optimum balance of FSH plus LH would tend to imply that the separation of the two factors leads either to a loss of ovulating potency or to the accumulation of inhibitory material. The large variability observed in these experiments rules out any definite decision. We have employed animals in prime condition and most of them are from a single chinchilla strain, but repeatedly we have found that two littermates of the same weight will differ markedly in the response to an identical series of injections. The one showing the lesser response has almost always exhibited a much smaller ovary and uterus at autopsy indicating a physiological underdevelopment in the generative organs not superficially apparent. For this reason we recommend for best results a 5 to 6 month old rabbit rather than a younger one. Those does giving all indications of being on the verge of sexual maturity have given the best responses.

In practically all of the mated animals fertilization of the superovulated ova has occurred. In some of the animals receiving the higher doses of mare's serum extracts (columns 12 and 18) sperm evidently failed to reach the ova. Further, in any large lot of eggs 1 or 2 out of 30 or more may be unfertilized. Finally, in an occasional animal in the FSH and AP series, sperm failed to reach the eggs. More puzzling is the fate of superovulated ova. A number of animals were sacrificed at 3 days after copulation. In some of these the number of ova recovered was the same as the number of ovulation points in the ovaries, but in others either a reduced number or no eggs were obtained. Careful flushing of tubes and uteri indicated that the ova had been completely expelled from the oviducts. This indicates that in certain animals the effect of the gonadotropic material is to induce oviducal contractions of a sort to expel the ova. This may be due either to faulty corpus luteum development or to a continued overbalancing production of oestrogen from the ovaries. The latter seems likely since the corpora lutea appear quite normal in most instances.

Superovulation in immature (25 day) rats after the administration of mare's serum extract has been reported by Cole ('36, '37). In his series only a single subcutaneous injection was used but roughly similar effects to those reported here were observed, e.g., a low dose (2 r.u.) led to ovulation of less than the normal number, increasing the dose to 6 to 10 r.u. led to superovulation in about 70% of the animals and a further increase to 16 r.u. led to superovulation in a similar percentage, animals receiving 500 r.u. failed to ovulate in all but one instance and then only 4 ova. The number of ova obtained varied widely also, but they were fertilized in a number of instances and living fetuses produced. The immature rat appears then to differ from the rabbit in being more responsive to mare's serum. Cole found that adult rats could not be induced to superovulate with mare's serum extracts. We have superovulated a number of adult rabbits (chiefly in the FSH series, see also column 48) and have observed in a few instances up to 36 living fetuses.

SUMMARY

A study was made of the ovulating effects of three extracts of mare's serum and three pituitary extracts. One hundred and seventy-three animals were used, 40 receiving mare's serum extracts and 133 receiving pituitary extracts. The data indicate that all the gonadotropic preparations will ovulate prepubertal or pubertal rabbits when properly administered, but that mare's serum preparation will induce superovulation only occasionally and then never to the extent of the pituitary extracts; the latter usually causes superovulation in the recipients. The pituitary extracts employed induced, on the average, ovulation of three to more than four times the normal number of ova to be expected from animals of this size. Up to eighty eggs have been obtained from single individuals. The difference between the mare's serum preparations and the pituitary preparations appears to be in the tendency of the former to produce cystic unovulable follicles. Superovulated ova are fertilizable, and mating does not seem to affect the number of eggs produced. Copulation alone cannot substitute for an ovulating injection in young rabbits given a series of priming doses. An unfractionated purified extract has proven more efficient in superovulating than a combined administration of FSH and LH.

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THE MORPHOLOGY OF THE NERVE TERMINALS OF THE HUMAN SPINAL CORD AS SEEN IN BLOCK SILVER PREPARATIONS, WITH ESTIMATES OF THE TOTAL NUMBER PER CELL

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ONE TEXT FIGURE AND ONE PLATE (SIX FIGURES)

INTRODUCTION

The earlier work on the morphology of the synapse of the human central nervous system was summarized by Lache ('06). In this report he refers to the human spinal cord as particularly suitable material for demonstrating nerve terminals² with the Cajal techniques. He described the end bulbs as small round or oval masses usually continuous with a terminal neurofibril. He noted that the centers of the endings often appeared light, due, presumably, to the failure of impregnation. Lache mentioned that the number, arrangement, and volume of the terminals varied greatly from cell to cell in normal, as well as in diseased, cords, and pointed out a difference in appearance of the endings in adult and infant.

In the more recent literature, nothing has come to my attention that enlarges upon the above description, although

¹ The work reported in this paper was done in the Department of Anatomy, University of Minnesota, under the direction of Dr. A. T. Rasmussen, and was submitted to the Graduate Faculty as a portion of a thesis in partial fulfillment of the requirements for the degree of doctor of philosophy. Dr. Edith Boyd, Department of Anatomy, University of Minnesota, supervised the quantitative work.

² The term, nerve terminals, is used synonymously with boutons terminaux, boutons, end feet, end bulbs, central nerve endings. The forms usually described as boutons en passant or as collateral swellings are referred to in this paper as pre terminal endings.

several brief notations on human nerve terminals have appeared (compare Hoff, '33; Duncan, '38). For additional literature on human boutons terminaux, see Cajal ('08 and '34) and Achucarro ('09); and for a description of end bulbs in the superior cervical ganglion of man, refer to Greving ('21).

The recent literature on the nerve terminals of the central nervous system in lower forms is abundant, but, because there will be occasion to refer to many of these papers later, no special survey of this material is indicated here.

The purpose of this paper is to describe the block silver picture of the end bulbs of the human spinal cord, and to correlate these findings with those reported in the literature. Estimates of the total number of nerve terminals per cell in the various nuclear columns of the spinal cord are included.

MATERIAL³ AND METHODS

Sixty-eight human spinal cords have been examined in this study. The subjects from whom the cords were taken range in age from newborn to 83 years. Of this series, six cases were 9 months old or younger and to these the following descriptions do not apply. In these younger specimens, although optimally obtained and prepared, the technique used failed to show nerve terminals comparable to those of the older groups. In addition, there were three cases among the specimens older than 9 months in whom no terminals were demonstrated at the levels examined. These spinal cords were in advanced stages of necrosis from disease processes, with uniform diffuse tissue destruction.

Gross technique

The spinal cords were obtained at routine autopsies by the following procedure. The vertebral bodies and intervertebral discs in the eviscerated subject were divided sagittally from

³ For assistance in obtaining material, the author is indebted to Dr. R. Brochner, Dr. C. B. Craft, and especially to Dr. L. Berman, Department of Pathology, University of Minnesota.

the lower lumbar to the upper thoracic segments. The pedicles on one side were then sawed through, permitting a chain of vertebral half-bodies to be lifted off, and leaving one-half the column intact. The nerve roots in the spinal canal were cut external to the dura. By dividing the cauda, the spinal cord and its membranes were removed from the canal as far superiorly as the upper thoracic region, at which point the dura was incised circularly around the cord. Through the foramen magnum the nerve roots of the cervical segments were cut internal to the dura, freeing the cord in the cervical canal. By gentle traction with forceps on the lumbar dura and cauda the spinal cord was pulled from the cervical canal and obtained in toto.

Microscopic technique

Barr ('39) used formalin perfusion fixation on cat material and adapted the reagent times of Cajal's technique (modification 4) to produce excellent preparations of spinal cord nerve terminals. Because of the consistent results with this technique, and its applicability to human material without perfusion, it has been used throughout in this study. Other fixatives have not improved the preparations and have been most unhandy in dealing with autopsy material. In my hands, the block silver method has been far more satisfactory in demonstrating nerve terminals in human cords than the protargol method as recommended by Bodian ('36, '37 b).

It has been found advantageous to cut the blocks for fixation and impregnation thicker than usually recommended. With a thick block, approximately 1 cm. in length, there is little danger of over-impregnation. Almost invariably an intermediate zone of desirable silver intensity may be found, minimizing the importance of time in the silver bath. In this manner a remarkably uniform series of sections may be obtained from a large number of blocks.

The usual limits of time for the various reagents applied to this material are: 1, 12% neutral formalin, 12 to 36 hours; 2, running water wash, 6 to 12 hours; 3, ammoniated alcohol

(10 drops of concentrated ammonium hydroxide in 100 cc. of 95% alcohol), 24 to 36 hours; 4, 1.5% silver nitrate at 37°C., 24 to 48 hours; 5, 2% pyrogallie acid (in dark), 12 to 24 hours; 6, dehydrate, clear, embed in paraffin and beeswax, and cut at 10 μ . Albumen fixative was used with forty-four specimens and starch fixative (Spoerri, '39) with the remainder. Several changes of 70% alcohol and of xylol are necessary to remove the excess reducing fluid. As mentioned by others, chemically clean glassware is requisite. The blocks may be left in silver longer than indicated above without damage (compare Davenport, '33), but with this material the best results have been obtained by removing the blocks when they are colored a tobacco brown, which usually is attained in less than 30 hours.

Method of observation

In the examination of the sections, all the nerve terminals found in the gray matter of one side were counted. This procedure was greatly facilitated by placing parallel hairs across the ocular diaphragm to define the field and avoid duplication. All measurements were made with an ocular micrometer callibrated in the usual manner with a stage standard.

In recording the terminals in relation to cell bodies and processes, the following nuclear columns were recognized: substantia gelatinosa Rolandi, dorsal sensory and internuncial, dorsalis (Clarke), intermediate sensory and internuncial (including substantia gelatinosa centralis), intermediolateral, ventral sensory and internuncial, anteromedian, posteromedian, anterolateral, posterolateral, central, retroposterolateral, accessory, phrenic, and the nuclei of Stilling (sacral and cervical).

OBSERVATIONS

Of the nuclear columns examined, nerve terminals were found around the cells of all except the nucleus substantia gelatinosa Rolandi. This finding agrees with that for the

spinal cord of cat (Barr, '39). Occasionally, cells are found in this location with end bulbs in relation to them but because of their configuration they are considered to be displaced dorsal column or posteromarginal column cells. Likewise, in the central gelatinous substance, nerve endings were seen only in relation to cells with the morphology of the intermediate sensory and internuncial cells, with which group they were placed in counting. Of the terminals around cells in the remaining columns, those in the intermediolateral cell group appear to stain less satisfactorily than elsewhere.

Morphology

The general appearance of the nerve terminals in the human cord is similar to that described repeatedly for lower forms. They appear as blackened swellings in a terminal or pre-terminal position along the axone ramifications (figs. 2 to 7). Most frequently they appear in contact with a cell body or robust process. In no instance in this material has there been any morphological evidence for continuity at the synapse. As a rule, no end bulbs are seen free in the neuropile. Almost invariably they occur adjacent to cell bodies or to those cell processes which have a greater girth than a fibrillar dendritic termination. A process less than $2.5\ \mu$ in diameter seldom has boutons in relation to it.

As the end bulbs were counted they were classified according to size and configuration into the following fourteen groups: 1, small regular loops, 0.5×1 to $1 \times 1.5\ \mu$; 2, large regular loops, 1.5×2 to $3 \times 4\ \mu$; 3, regular filamented loops, 1×1.5 to $3 \times 4\ \mu$; 4, fibrillated bulbs, 1.5×2 to $3.5 \times 5\ \mu$; 5, thick small loops, 0.5×1 to $1 \times 1.5\ \mu$; 6, thick large loops, 1.5×2 to $3 \times 4\ \mu$; 7, thick filamented loops, 1×1.5 to $3 \times 4\ \mu$; 8, thick fibrillated bulbs, 1.5×2 to $3.5 \times 5\ \mu$; 9, granular small loops, 0.5×1 to $1 \times 1.5\ \mu$; 10, granular large loops, 1.5×2 to $3 \times 4\ \mu$; 11, granular filamented loops, 1×1.5 to $3 \times 4\ \mu$; 12, granular fibrillated bulbs 1.5×2 to $3.5 \times 5\ \mu$; 13, opaque masses, 1×1.5 to $3 \times 4\ \mu$; 14, granular masses, 1×1.5 to $3 \times 5\ \mu$.

It is to be noted that there is considerable overlap in size among the various types. In this respect the human terminals appear to differ from those of cat in which Gibson ('37) and Barr ('39) describe differences in configuration following differences in size. They had, however, fresher material.

By rearranging the above groups, the terminals fall into five primary types (small loops, large loops, filamented loops, fibrillated bulbs, and opaque or granular masses) each of

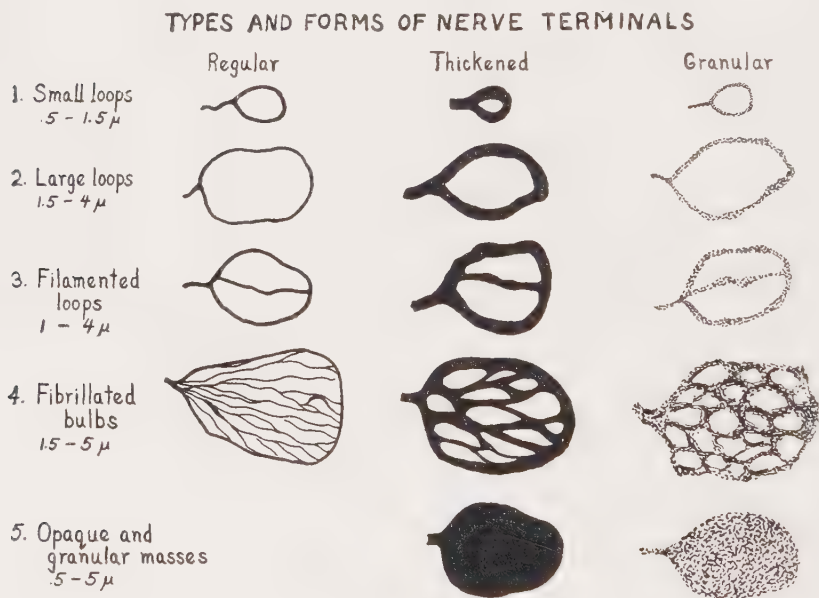


Figure 1

which may appear in three forms (regular, thickened or granular). This classification is presented diagrammatically in figure 1. For descriptive convenience the numbered primary groups will be referred to as "types" and the subgroups (regular, thickened, granular) as "forms." These diagrams are drawn as multiples of average terminals to illustrate comparative sizes. The measurements given are of the longest dimensions.

Nearly all these types and forms of terminals seen in block silver sections of human cords have been described in lower forms. Each has been variously interpreted. The regular small and large loops probably represent the typical end bulbs. Of these, the small loops have been assigned special relationships on Mauthner's cell by Bartelmez ('15). Bodian ('37 a) claims that the appearance of loop structures is peculiar to block silver preparations, and that the light center is probably due to the failure of impregnation of the central core (compare Lache, '06). Hoff ('32) describes the loops as the "normal" ending, the other configurations representing degenerate bulbs. A species predominance of loops is noted for chicken, rat and rabbit by Phalen and Davenport ('37). Marui ('18) mentions this type of terminal but groups them with other end bulbs as swellings along axone fibers where dissolution takes place.

The thickened loops are described by Hoff ('32), and by Gibson ('37) as early degenerating forms following transection of the axone, but Phalen and Davenport ('37) find these forms as normal constituents in an extensive group of animals. Barr (personal communication) considers granular loops a result of technical difficulties. These forms have been described as a result of experimental degeneration by Marinesco ('04) and by Hoff ('32).

To Hoff ('32), terminals with central cores or filaments represent the typical condition in the 24-hour stage of degeneration. He also indicates that this type is sometimes seen in unoperated animals. Barr ('39) ascribes no special significance to it. Bodian ('37 a), using cytological technique, described a small end bulb with a central core of mitochondria surrounded by a thin film of neuroplasm, but these, he claims, will show as delicate rings with silver. No specific reference in the literature to thickened and granular forms of filamented loops has come to my attention.

The regular fibrillated bulbs of the human material seem to correspond to the basket-like terminals mentioned by Phalen and Davenport ('37) for dog and pig. The reticular bulb

of Cajal is apparently the same ending, and it is quite probable that these correspond to Hoff's early degenerating bulbs. Although Marui ('18) refers to them as optical illusions, he describes similar swellings with a neurofibrillar internal structure in relation to Mauthner's cell. These may represent the silver picture of the large club-endings of Bartelmez ('15). Snider ('36) describes fibrillated terminals in the cerebellar cortex of cat. Held's fibrillar endfeet (1897) and the fibrillated terminals mentioned by Lache ('06) are likewise similar to this type of bouton. Windle and Clark ('28) illustrate such endings in their drawings of the spinal cord of the various animals examined by them.

The thickened form of the fibrillated bulb is relatively rare in the human cord. These might well correspond with more advanced degenerating bulbs in Hoff's material, and they fit the description of the intermediate degenerating forms of Gibson ('37), who pointed out that increased silver affinity in end bulbs is indicative of deterioration. In this series, the form of fibrillated bulb described as granular is similar to the vessiculated bulb of Shimert ('38) and the irregular, reticulated, degenerating terminal described by Foerster, Gagel and Sheehan ('33).

Of the two forms of type 5, the opaque one has been pictured by Marinesco ('04) as a swollen degenerated terminal. He also describes opaque masses as components of the normal synapse. These forms are interpreted as fairly advanced degenerating terminals by Hoff ('32) and by Gibson ('37). Snider ('36) describes opaque forms but sees no reason to consider them as degenerating. Bodian ('37) believes these forms are completely blackened by silver impregnation, while Windle and Clark ('28) attribute them to an optical effect due to their position on the side of the cell. Tiegs ('31) believes these forms to be tangentially cut sections of neighboring unmyelinated fibers.

The granular forms of type 5 in this material are the structures referred to by Hoff ('32) and by Gibson ('37) as the end product of bouton degeneration. Granular debris fre-

quently appears on the cell surfaces (compare Bodian, '37 a), but such deposits may be distinguished from a granular terminal by the absence of a neurofibril attachment and by general conformation and relation to cell body or process.

From the above discussion it is evident that there is considerable disagreement with regard to recognition and interpretation of the various morphological types of nerve terminals. In view of the reports of altered nerve terminals resulting from disease processes and experimental procedures, it is obvious that there should be agreement as to what should be regarded as normal, and what as abnormal, end bulbs. In order to establish some working distinction, technique effects must be recognized, autolytic changes taken into account, age differences analyzed for the species under consideration, and regional differences in a single specimen determined. With this information at hand, an altered bouton picture could be demonstrated by comparison to the normal standard for the same age under the same conditions of technique and autolysis. In order to do this it would seem that some quantitative expression of the relationships of nerve terminals one to another must be available. Closely related to this problem is that of determining the total number of boutons ending in relation to a single nerve cell. This information has a definite bearing on concepts of synaptic transfer of impulses (compare Liddell, '34; Eccles, '37) as well as on an understanding of the anatomical nature of the synapse. The remainder of this paper will be concerned with this phase of the problem. The quantitative data illustrating the normal variation in end bulb morphology, the influence of age, autolysis, technique, and pathology on the bouton picture, and the significance of the types and forms of terminals will be presented in a later paper.

The number of end bulbs per cell

In the face of two primary difficulties—the erratic nature of silver techniques, and the need of reliable estimates of nerve cell surface area—several attempts have been made to

state the number of boutons per cell appearing in lower forms. In *Ameiurus*, Bartelmez and Hoerr ('33) counted a maximum of forty-two large, and eight small, club endings around Mauthner's cell. Bodian ('37 a) has extended these observations, and, claiming that all boutons adjacent to Mauthner's cell were preserved, gives the following figures: numerous tiny end feet on the axon cap; 100 or more large end feet on the cell body; 100 to 200 large end feet on the ventral dendrite; 30 small, and 90 large, clubs on the distal part of the lateral dendrite; and numerous tiny end feet and 100 to 150 large end feet on the proximal part of the lateral dendrite. Lorente de Nó ('34) estimates that 40 to 50 end bulbs terminate in relation to a single pyramidal cell of the third layer of the mouse cortex, and indicates that several hundred collateral terminals may end in relation to a single dendrite. In the comparative material of Phalen and Davenport ('37) the number of terminals per cell varied so greatly that no attempt was made to count all, but from random selection of cord cells they give the following figures: none in fish, urodele, frog, and fetal calf; occasional bulbs in turtle, duck, pigeon, chicken, and rat; 1 to 5 on most cells in rabbits; in pig, sheep, and monkey, 5 to 10 per cell; and over 10 per cell in cow, dog, and cat. These figures are probably for cell sections rather than for serial counts through an entire cell.

For terminals in the spinal cord of cat, Davenport ('33) reports variations from a few to several hundred per cell. Hoff ('32), using chloral hydrate perfusion, claims to have stained all the boutons in the adult cat cord. He counted 100 terminals in an area of $20.5 \times 62.7 \mu$, or 1 bouton per $12.8 \mu^2$. With this method, Hoff estimates a range for perikaryon density of 1 terminal per $9 \mu^2$ to 1 per $17 \mu^2$. He states that the density on the dendrites is of the order of 1 terminal per $20 \mu^2$. On this basis Hoff estimates a total of 300 to 350 boutons per ventral horn cell in the cat. He also gives figures for the cord cells of a monkey (18 months old) which, he states, may not yet have its full quota of boutons: Clarke's column cells, 20 to 50 terminals; dorsal horn cells, numerous tiny

loops; ventral horn cell density, 1 terminal per $18 \mu^2$. In the cat cord, Barr ('39) finds an average density of 7 end bulbs per $100 \mu^2$. In selected sections he counted a maximum of 24 boutons per $100 \mu^2$ and an average of 16 per $100 \mu^2$. Assuming the nerve cell to be a sphere, for calculating surface area, he estimated that 1250 terminals ended in relation to a ventral horn cell body. With those along the dendrites included, he suggests a value of several thousand per total ventral horn cell of the cat. His data indicate that about 38% of the cell surface is engaged in synaptic contact.

The findings for the human spinal cord, summarized from counts of over 35,000 end bulbs, are presented in table 1.

TABLE 1

NUCLEAR COLUMN	BOUTONS PER CELL SECTION (AVERAGE)	BOUTONS PER $100 \mu^2$	BOUTONS PER CELL BODY (COMPUTED)	BOUTONS PER CELL (SERIAL COUNTS)
Dorsal sensory	2.0	16.3	165	88
Intermediate sensory	3.2	13.2	269	137
Ventral sensory	3.3	14.2	144	112
Dorsalis	4.8	14.4	723	283
Intermediolateral	2.6	18.7	367	97
Posteromedian	6.7	17.3	665	312
Anteromedian	6.4	27.6	1755	493
Posterolateral	6.9	28.8	1832	833
Anterolateral	6.3	23.2	1475	658

The first column gives the average number of terminals per cell section for the entire series in the nuclear columns indicated. The counts used in obtaining these averages varied from no terminals to 170 per cell section. It is to be noted that these figures are for cell sections, including every cell fragment, and not for an entire cell. The figures of the second column represent counts similar to those of Hoff ('32) and Barr ('39) for the cat. In these, no correction was made for the curvature of the cell surface, and only optimal areas, arbitrarily defined, were counted. The error in such an estimate will be larger for the columns with small cells because of the greater curvature. The third column figures represent the computed number of terminals per cell body, assuming uni-

form distribution of terminals over the surface and the shape of the cell to be spherical. This geometric figure was adopted for want of a more accurate expression of the surface area of these nerve cells. On the basis of the work of Krause and Weil ('26) on the shape of human nerve cells such an assumption is, of course, seriously inadequate. It has been used by Barr, however, as a rough approximation, and these data on human material are offered for comparison. The figures of the last column are from serial counts through optimal cells, including their processes as far as they could be followed.

Assuming that no staining peculiarities for different cell groups exist, the figures in the first column indicate that relatively more terminals end in relation to cells of the ventral horn, somewhat fewer in relation to cells of Clarke's column, and the least number in relation to the cells of the sensory and internuncial groups and the intermediolateral column. It is reasonable to suspect that this difference is related to the size of the cells concerned (compare Barr, '39).

From the figures for bouton density in the second column of table 1 it is seen that it varies from 13 to nearly 29 per 100 μ^2 . It is also evident that the number of terminals per unit area increases disproportionately on the motor cells. If these values were corrected for surface curvature the density distribution would become more disproportionate in favor of the motor cells because of the greater correction factor for the small cells. It is to be noted, too, that the bouton density on the cells of Clarke's column is of the same order as on the other sensory cells, although they have approximately the same linear dimensions as the ventral horn cells. Although the number of terminals per cell section follows cell size, it would seem that the density of the terminals has a definite relationship also to cell function, being greatest on the cells of the final common path. The relatively high bouton density for an optimal cell surface in the intermediolateral column in comparison with its low average number of boutons per cell section strongly suggests a difference in the staining for this column. This is supported by Cajal sections through the oculomotor nucleus, where visceral efferent cells are

close to somatic efferent, for the small visceral cells may be distinctly less strongly impregnated than the somatic.

The computed figures for boutons per cell body (column 3 in the table) are included to compare with similar figures reported for lower forms. The method used in the computation does not, however, permit a reliable estimate of such values. Adding the surface of cell processes and of irregularities on the cell body, these figures would be multiplied several times, probably to the order of several thousand boutons per ventral horn cell (compare Barr, '39).

The figures for the serial counts in table 1 are the maximum for any cell counted in the column indicated. In each case the figure falls far short of the computed number per cell body. It is necessary, then, to conclude either that optimal silver preparations fail to demonstrate the actual number of terminals, or that the density of the boutons over the cell surface is not uniform.

From the great variation in the density of terminals reported in the literature, and evident in this material, it would seem that bouton density is not uniform over a cell surface. With this supposition the computed values of terminals per cell would be lowered considerably. The further solution of this problem awaits assurance of uniform technical results. Because it is unsafe to assume that all terminals have been stained, even in optimal preparations, the serial count values presented here may be somewhat low. On the basis of these qualifications it may be that the actual number of terminals per cell in the human cord is somewhat below the computed values given, and probably above the values obtained from optimal serial counts. The latter values are also limited by the technical difficulty involved in following processes through complete serial sections. One thing that should be noted particularly is the favorable comparison between the number of boutons per 100 μ^2 surface area of the cells of this human material and that of animals in which the tissue may be obtained immediately after death. This lends assurance to the use of these figures as rough norms for gauging pathological changes.

SUMMARY AND CONCLUSIONS

1. In block silver preparations of sixty-eight human spinal cords, nerve terminals were demonstrated in all but nine. Of these nine, three were necrotic specimens and the remainder infant cords. The technique used failed to demonstrate terminals in cords of subjects under 9 months old.

2. No terminals were found in relation to the cells of the nucleus substantia gelatinosa Rolandi, but were evident in all other cell columns.

3. The terminals, as they appear in the human spinal cord, were classified according to size and configuration into five primary groups—small loops, large loops, filamented loops, fibrillated bulbs, and opaque or granular masses; and into three subgroups—regular, thickened, and granular. End bulbs of all types were noted in both terminal and pre-terminal positions along the axone ramifications.

4. While some of the above types may be artifacts produced by the silver impregnation technique, they may serve some useful purpose as criteria for evaluating experimental and pathological conditions, if they are regularly present in post mortem material of apparently normal specimens.

5. The size of the terminals varies from $0.5 \times 1 \mu$ to $3.5 \times 5 \mu$. There is considerable overlap in size among the several morphological types.

6. Averages for the entire series show that the number of boutons per cell section in the material and by the technique involved varies from 2 (sensory and internuncial column) to 6.9 (posterolateral column). These figures suggest that the absolute number of endings in relation to a cell is largely a function of cell size.

7. Counts of optimal preparations indicate that the density of boutons on a cell surface is related to function. The average density computed from maximum counts on motor cells of the final common path is 23.1 terminals per $100 \mu^2$ compared to 14.5 per $100 \mu^2$ on cells of the sensory columns.

8. Assuming uniform distribution of terminals over a single spherical cell surface, the boutons per cell body were estimated to be from 144 on small sensory cell bodies to 1832 on

the large cell bodies of the posterolateral column. With dendrite surfaces added these values would be of the order of several thousand per ventral horn cell.

9. Maximum serial counts through optimal cells ranged from 88 per cell of the dorsal sensory column to 833 per cell of the posterolateral column. These figures are considered more representative than the computed values; but, because of the difficulty in following processes through serial sections, and the insecurity of the assumption that all terminals have been stained, the serial counts are probably somewhat low.

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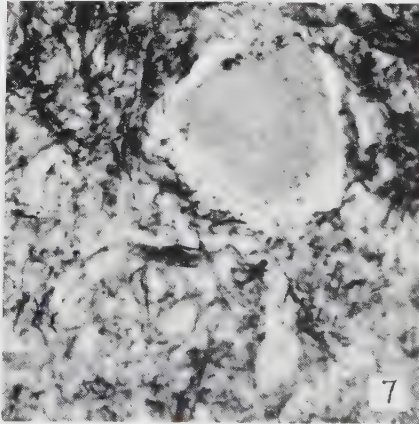
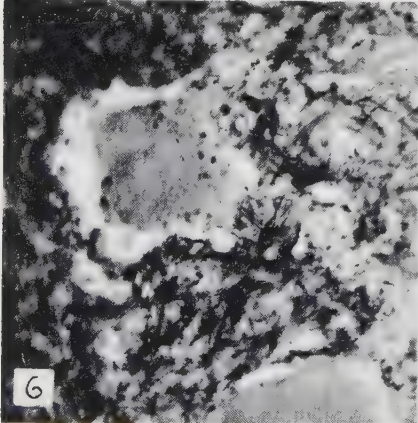
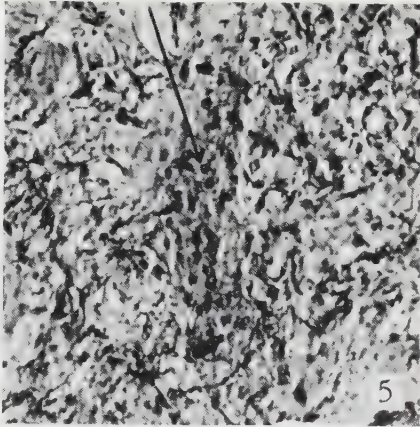
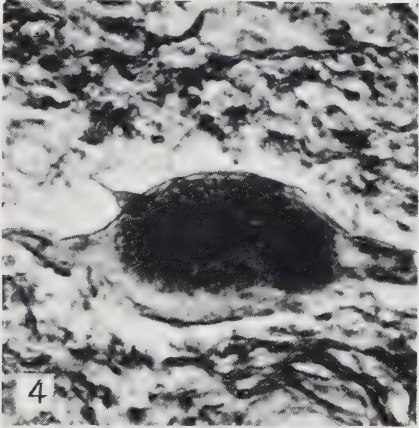
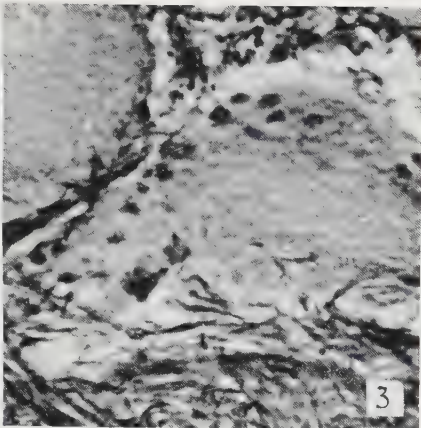
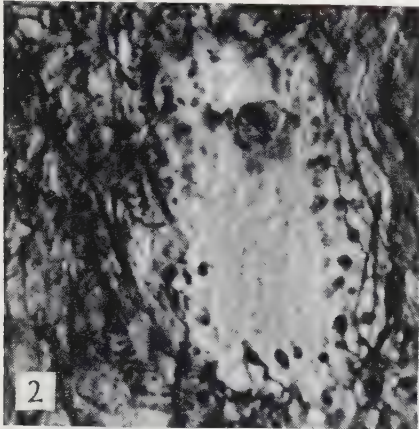
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PLATE 1

EXPLANATION OF FIGURES

Photomicrographs of nerve terminals adjacent to cell bodies and processes in the human spinal cord. Figures 1 to 4 are at a magnification of $\times 975$. Figures 5 and 6 are at a magnification of $\times 600$.

- 2 End bulbs and pre-terminal endings in relation to a cell of the posteromedian column.
- 3 Granular terminals on a cell of Clarke's column.
- 4 Large fibrillated bulb in relation to a cell of the posteromedian column.
- 5 End loops and opaque forms on a surface of a dorsal sensory cell.
- 6 End bulbs on a cell of the anterolateral column showing extension of terminals along the dendritic roots.
- 7 Nerve terminals in relation to a cell of the posterolateral column.



A CASE OF HYPER-ROTATION OF THE COLON

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ONE FIGURE

Instances of left-sided caecum and appendix are not uncommon. In all cases reported they have been due either to abnormal development of the mesentery or to reversed or arrested rotation. In the former case there is normal rotation of the bowel followed by failure of adhesion of the mesentery. This may be complete and leave a fan-shaped mesocolon (mesenterium commune) or be partial with an ascending mesocolon. Both result in a mobile caecum which may migrate to the left side. Reversed rotation may occur in the form of situs inversus which, of course, results in a permanent left caecum, providing mesenterial adhesions are normal. Left-sided caecum due to arrested rotation is found when the fetal umbilical hernia fails to rotate as it returns to the abdominal cavity. Here all portions of the colon lie behind and to the left of the small bowel (Keith, '33).

The left-sided caecum and appendix to be described here was not due to any of the above causes. It was due to hyper-rotation of the colon. Larimore's definition of hyper-rotation is used here. He states (Larimore, '26) that the term is used inclusively to denote the embryological movements of the gut from their original position in the midline to their final position. This begins with the rotation of the herniated umbilical loop of intestine. When the loop returns in the third month the caecum assumes a position inferior to the liver in front of

¹ Grateful acknowledgment is made to J. P. Elliott and S. L. Parker, medical students, for valuable assistance in the photography and dissection.

or slightly above the iliac crest. The second phase of the rotation consists of the descent of the caecum into the right iliac fossa. Dott ('23), using the twisting of the superior mesenteric artery as a basis of measurement, states that the normal rotation amounts to 270° .

DESCRIPTION

The subject was a middle-aged male Negro of sthenic type of build. The abdominal contents were healthy, no adhesions being present. The transverse, descending, and sigmoid portions of the colon and the rectum were normal. The ascending colon possessed no mesentery. From the hepatic flexure to the right iliac fossa it was normal. From here it descended into the pelvis minor, still retro-peritoneal. Folds of peritoneum extended from its superior and inferior borders to the posterior-superior surface of the bladder, presenting falciform margins directed to the left. They were continued to the right lateral pelvic wall to be attached there about 3 cm. below the brim of the pelvis minor. The colon turned here and ascended into the left iliac fossa. It continued vertically upward with the tip of the caecum located in the umbilical region at the level of the third lumbar vertebra. The appendix was normal and lay to the right of the caecum at the level of the fourth lumbar vertebra. The ileocaecal junction lay inferior to the appendix opposite the fifth lumbar vertebra. The caecum was of normal size. This portion of the colon lay between the vertebral column on the right and the descending colon on the left. The portion from the flexure in the true pelvis to the tip of the caecum was free except for the fold of peritoneum extending between it and the terminal portion of the ileum. The latter was, of course, drawn to the left to join the caecum and had drawn the root of the mesentery somewhat to the left so that its attachment was nearly vertical.

Dissection revealed that the anastomosis between the superior mesenteric (middle colic) and the inferior mesenteric (left colic) arteries occurred in the normal position in the transverse colon. The right colic and ileocolic arteries arose

from a common trunk and appeared normal except for an increase in the number of branches. The appendicular artery was normal. The superior mesenteric artery had undergone a torsion of approximately 400° against the normal of 270° .

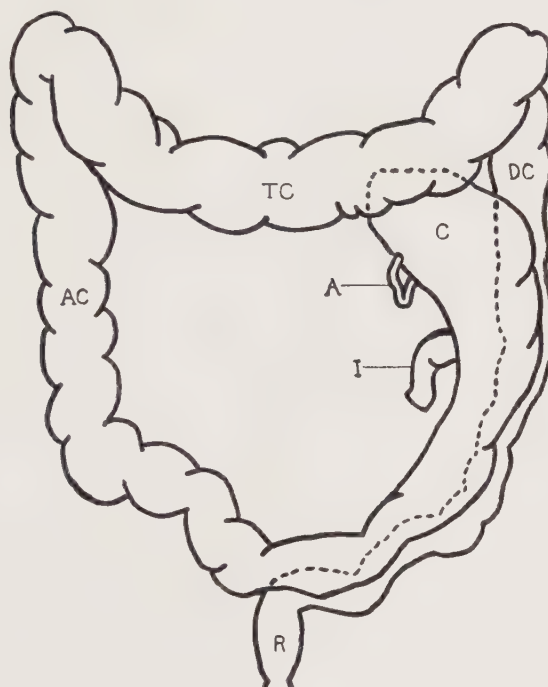


Fig. 1 Front view of large intestine (drawn from photographs). A, appendix; AC, ascending colon; C, caecum; DC, descending colon; I, ileum; R, rectum; TC, transverse colon.

DISCUSSION

The normal anatomy of the transverse, descending, and sigmoid portions of the colon indicates supernormal growth of the ascending colon as an explanation of the anomaly. This interpretation is supported by the normal position of the anastomosis between the right and left colic arteries. This arterial pattern indicates that the rotation of the gut upon its return from the umbilical cord was normal. Hyper-rotation

in this early stage would have drawn the left colic artery further to the right and possibly even some distance down the ascending colon. The normal size of the caecum seems to localize the growth somewhere between the ileocaecal junction and the hepatic flexure.

This case is unique insofar as we have been able to determine through search of the literature. Examination of reports covering more than 7000 post-mortem and barium examinations has revealed no similar case.

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THE EXPERIMENTAL SHORTENING OF DELAYED PREGNANCY IN THE ALBINO RAT

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THREE PLATES

The normal gestation period of the albino rats in our colony is 22 days but occasional individuals may have 21-day or 23-day gestation periods. Long and Evans ('22) first discovered that in the rat oestrus and ovulation occur shortly after parturition, usually within 24–36 hours. It does not seem to be very generally known, however, that if copulation and conception occur during this post-partum oestrus the gestation period which follows and during which the young of the first litter are being suckled, may be prolonged and extend over a period ranging from 22 to 36 or more days.

Pinard ('05) apparently was the first investigator to report the occurrence of prolonged gestation or delayed pregnancy in rodents. In some of his cases the period of gestation was half again as long as the normal duration. Daniel ('10) reported the occurrence of delayed pregnancy in lactating mice and stated that gestation was prolonged by 1 day for each mouse being suckled. King ('13) reported similar observations on the albino rat. These early workers attributed the prolongation of gestation to nutritive disturbances and implied that the suckling young received nutrition at the expense of those developing *in utero*, thus preventing them from growing at the normal rate.

Kirkham ('16 a, b, '18) working with mice discovered that ovulation and fertilization in the majority of cases occur normally during the post-partum oestrus of lactating females. He reports a rather high percentage of failures, however.

Segmentation of the fertilized eggs and their passage down the Fallopian tubes occurs at the same rate in both nursing and non-nursing mothers. Kirkham found that the prolongation of gestation was due to delayed implantation and stated that the fertilized eggs of lactating females lie free in the lumen of the uterus from the sixth to the fourteenth days. He believed that the uterine cells would not react to the blastocysts and enable them to become implanted before the fourteenth day. He interpreted this as being due to a withdrawal of nourishment from the parent by the suckling young. Kirkham also noted that the embryos in the uteri of pregnant females suckling the same number of young did not always develop at the same rate. He attributed this to individual variation in the influence of the mammary glands.

Mirskaia and Crew ('30) report that the degree of prolongation of gestation in lactating mice is variable but that it has no relation to the number of young in the uterus nor to the number suckling. They do not find any support to the contention that there is some inhibitory action exerted by the mammary gland. They suggest that delayed implantation and prolonged pregnancy are due to the inability of the corpus luteum to cater adequately for implantation and lactation at the same time. Hain ('34) also finds that when the pregnant albino rat is suckling pregnancy is prolonged but he reports that its duration bears no relation to the number of young being suckled.

Enzmann, Saphir and Pincus ('32) confirmed Kirkham's observation that prolonged gestation in mice is due to delayed implantation. They found, however, that the time of implantation in inseminated lactating mice does not always occur on the fourteenth day but may take place any time between the sixth and sixteenth days and that the time of implantation depends on the number of suckling young. Furthermore, they state that each of the suckling young delays the ensuing parturition 21 hours rather than 1 day as reported by Daniel. These investigators also report that very little, if any, growth takes place in the blastocysts as they lie free in the uterine

lumen but that after implantation finally occurs the growth curve of the developing young is indistinguishable from that of an embryo during normal pregnancy. The birth weight of young from a prolonged gestation is the same as the weight of young born at normal term. Detailed data are given in another paper (Enzmann, '35).

Hamlett ('35) reviewing the entire subject of delayed implantation and discontinuous development in mammals, suggests that the delay may be due to an alteration of hormonal conditions in lactating females rather than to such a simple phenomenon as withdrawal of nourishment through the mammary glands. He believes that the corpus luteum through its influence on the uterus plays an important rôle in delayed pregnancy. Since the activity of the corpus luteum depends on the hypophysis, alterations in hypophyseal activity may account for all of these unusual findings. Hamlett reports that attempts to shorten the free vesicle stage by the use of hypophyseal extracts or other hormones (luteal extracts, Antuitrin-S) have completely failed. Pincus ('36) by injecting oestrone-free corpus luteum extracts into lactating pregnant mice also failed to bring about the desired results.

It seemed to the writer that in the light of recent endocrine discoveries the entire problem was worth reinvestigating. The albino rat rather than the mouse was chosen as a subject since so few observations have been made on that animal. He wishes to thank Dr. Elizabeth Ward for permission to include some data gathered by her, in this paper. He is indebted to his wife for her technical assistance and constant help in making daily observations of the experimental animals. Mr. Anselm Schurgast very kindly assisted in the preparation of the plates. The writer wishes to take this means to express his appreciation to Dr. Oliver Kamm of the Parke-Davis Laboratories for his interest in contributing a generous supply of Antuitrin-S for this work and to Dr. Max Gilbert of the Schering Corporation for supplying liberal quantities of Proluton (crystalline progesterone in sesame oil).

PRELIMINARY OBSERVATIONS

In some preliminary work, observations were made on the length of fifty-six gestation periods in rats suckling various numbers of young. These data were obtained by Dr. Elizabeth

TABLE 1

Variations in the length of gestation period in pregnant rats suckling 1-12 young

NUMBER OF CASES	NUMBER OF YOUNG SUCKLING	GESTATION (DAYS)
4	1	22
3	2	22
2	2	23½
2	3	24
3	3	25
1	4	25
3	4	26½
1	4	28
3	5	26
2	5	26½
2	5	27
2	6	27
3	6	28
1	7	27½
2	7	28
2	7	28½
3	7	29
2	8	31
3	9	32½
2	9	33
1	9	33½
3	9	34
2	10	33
1	10	34
2	10	35
1	12	36

Ward. Her figures which are given in table 1 have been supplemented by some obtained by the author. In all of these cases spermatozoa were found in the vagina within 24-36 hours after parturition. The male was then removed from the breeding cage.

Examination of the above table shows clearly that the more young that are suckling the greater is the prolongation of the

gestation period. Discrepancies occur but the general trend is unmistakable.

In order to study the conditions of the reproductive organs during delayed pregnancy, forty-two inseminated lactating rats which were suckling various numbers of young, were sacrificed on the day before parturition would be expected to occur (twenty-first day of gestation). Ten of these were not pregnant. This fairly high percentage of negative results is in keeping with reports of other investigators who believe that in many cases ovulation fails to occur at the post-partum oestrus or else the fertilized eggs die prior to implantation. It may be too, that the spermatozoa sometimes fail to reach the upper limits of the Fallopian tubes. Typical pregnant uteri were selected to be photographed and are shown in plate 1 (compare with plate 3, fig. H).

Although in this series of thirty-two uteri, discrepancies occur, nevertheless it is clear that on the twenty-first day of gestation, the greater number of young being suckled, the smaller are the developing embryos.

Careful examination of the ovaries of the thirty-two rats obtained in this series revealed that the corpora lutea of those suckling large numbers of young are definitely smaller than those of rats suckling smaller numbers (see plate 2). In order to obtain definite quantitative data on the size of the corpora lutea, the diameters of eight corpora lutea chosen at random from each animal were measured with a Vernier caliper and an average taken. The data are presented in table 2.

Again, discrepancies occur but there can be no doubt as to the trend. It appears that there may be a minimum size for the corpora lutea, for there is less variation among those suckling large numbers of young than among those suckling smaller numbers.

These observations suggested that perhaps when large numbers of young are being suckled the delay in implantation may be directly related to the fact that the endometrium is not properly prepared for the reception of the fertilized ovum and that failure of a progestational endometrium to appear at the

normal time may be due to insufficient amounts of progesterone being secreted by the smaller corpora lutea.

Accordingly some experiments were undertaken in an attempt to bring about implantation at the normal time by properly preparing the endometrium in rats suckling between six and thirteen young.

TABLE 2
Variations in diameter of corpora lutea in pregnant rats suckling 1-12 young

NUMBER OF YOUNG SUCKLING	AVERAGE DIAMETER OF CORPORA LUTEA
0	2.261 mm.
1	2.025 mm.
2	2.025 mm.
3	2.012 mm.
4	1.931 mm.
5	1.643 mm.
6	1.637 mm.
7	1.654 mm.
8	1.562 mm.
9	1.556 mm.
10	1.437 mm.
11	1.487 mm.
12	1.425 mm.

EXPERIMENTAL

Seventeen inseminated lactating rats were injected intramuscularly with various amounts of crystalline progesterone in sesame oil (Proluton; Schering Corporation) and for varying lengths of time. All animals were sacrificed on the twenty-first day after spermatozoa were found in the vagina. Of these, nine failed to become pregnant. The data for the remaining eight are included in table 3.

Injections of small quantities of another corpus luteum preparation (Lipo-lutin; Parke-Davis) gave similar results in two out of three cases.

These observations are interesting because they indicate that small quantities of progesterone are insufficient, under the conditions described here, to prepare the endometrium of inseminated lactating rats for an early and normal implanta-

tion. Large dosages (1 to 3 mg. daily) seem to be required but the length of time over which such injections should be administered has not as yet been determined. This is in keeping with the results of other investigators who find that relatively large doses of progesterone are required to inhibit normal oestrus and ovulation (Selye, Browne and Collip, '36; Lahr and Riddle, '36, and Phillips, '37).

In these experiments in which progesterone alone was administered no fully developed living foetuses were found on the twenty-first day of gestation (see plate 3). The percentage

TABLE 3

Results of injecting various amounts of progesterone for different lengths of time in rats suckling 6-13 young. Acceleration refers to time of implantation when compared with uninjected rats suckling similar numbers of young

RAT	NUMBER OF YOUNG SUCKLING	AMOUNT PROGESTERONE GIVEN AT EACH INJECTION	DAYS ADMINISTERED	RESULTS
		<i>mg.</i>	<i>days</i>	
D 255	11	0.1	20	Slight acceleration
D 275	6	0.2	3-5-7-9	Some acceleration
D 297	10	0.2	3-5-7-9	No acceleration
D 251	9	0.2	8	Some acceleration
D 296	13	0.5	3-5-7-9	Slight acceleration
D 294	10	0.5	8	No acceleration
D 328	10	1.0	20	Good acceleration but death and resorption
D 335	12	3.0	20	Marked acceleration but death and resorption

of animals in which pregnancy failed to occur is considerably higher in these experimental animals than in the normal controls.

The next procedure was an attempt to stimulate the secretion of the animals' own corpora lutea by injecting some luteinizing gonadotropic agent. Accordingly twenty-five inseminated lactating rats were injected for different lengths of time with various amounts of Antuitrin-S. All were sacrificed on the twenty-first day after spermatozoa were found in the vagina. The data for this experiment are given in table 4.

The results show that daily injections of 20 rat units of Antuitrin-S for the first 7 or 8 days after insemination are effective in bringing about implantation at the normal time in these lactating rats. In one case similar results were obtained when 20 rat units were injected daily for 20 days.

TABLE 4

Results of injecting various amounts of Antuitrin-S for different lengths of time in inseminated rats suckling 6-12 young

RAT	NUMBER OF YOUNG SUCKLING	AMOUNT ANTUITRIN-S GIVEN AT EACH INJECTION	DAYS ADMINISTERED	RESULTS
		<i>rat units</i>	<i>days</i>	
D 240	6	10	8	Negative
D 216	12	10	20	Negative
D 257	9	20	first day	Negative
D 249	9	20	first day	Negative
D 261	8	20	2	Negative
D 256	7	20	2	Negative
D 259	6	20	4	Negative
D 244	8	20	4	Negative
D 260	6	20	4	Negative
D 254	11	20	6	Negative
D 258	10	20	6	Delayed implantation
D 302	10	20	7	3 full term foetuses
D 250	8	20	8	1 full term foetus
D 221	8	20	8	2 full term foetuses
D 276	11	20	8	1 full term foetus
D 248	8	20	8	Pregnant but resorbing
D 222	8	20	8	2 full term foetuses
D 215	11	20	20	3 full term foetuses
D 232	9	20	20	Negative
D 228	9	50	20	Pregnant but resorbing
D 229	7	100	20	Negative
D 264	9	20	3rd day only	Negative
D 263	10	50	3rd day only	Negative
D 265	8	100	3rd day only	Negative
D 266	12	20	5-6-7-8	Delayed implantation

Although fully developed viable foetuses were present in the uteri of these animals on the twenty-first day of gestation, nevertheless none of the pregnant uteri appeared normal. The number of foetuses was always small and resorption areas were common (see plate 3). Apparently the stimulated ovaries

with their large numbers of corpora lutea were not adequate to support the continuous development of more than a few embryos even after normal implantation had occurred. The experiment shows, however, that a gonadotropic luteinizing hormone may be effective in bringing about implantation at the normal time in inseminated rats suckling fairly large numbers of young.

Since even larger doses of Antuitrin-S failed to produce normal appearing uteri, it was decided to supplement Antuitrin-S injections with small doses of progesterone.

Accordingly nine inseminated lactating rats suckling eight to twelve young were injected daily for the first 8 days with 20 rat units of Antuitrin-S. Following this period of injection each rat was injected intramuscularly every other day with 0.2 mg. of progesterone. Two of these failed to become pregnant. Five of the remaining seven were sacrificed on the twenty-first day. Of these, four had uteri that were indistinguishable from a normal uterus at term (see plate 3, I; compare with H). They contained two, two, five and eight fully developed living foetuses respectively. The fifth rat had but one living foetus and one resorption area. The two remaining rats were permitted to go to term. D 314, suckling ten young was delivered of two normal living offspring early on the twenty-third day of pregnancy. These young died after 4 days, apparently from malnutrition. The mother retained her maternal instincts for she continued to retrieve and cuddle the young but seemed incapable of nursing them. D 311, suckling eleven young, delivered four offspring toward the end of the twenty-second day, 13 to 14 days earlier than would have been the case had no injections been given. One of these lived for only 12 days but the other three were successfully reared and appeared normal in every respect except that their eyes opened 2 days earlier than the average for our colony.

Thus, it has been demonstrated that when proper hormonal conditions are supplied it is possible to bring about normal implantation, gestation, delivery and rearing of young in inseminated lactating rats suckling large numbers of young.

In some additional and similar experiments, nine rats injected with estrogenic substances (theelin; stilboestrol) or pregnant mare serum gave only negative results.

DISCUSSION

The mechanism controlling delayed implantation in inseminated lactating rats is undoubtedly complicated. The experiments cited above throw some light upon the problem and indicate that hormonal imbalance is the *raison d'être* for the phenomenon.

It is well known at the present time that ovariectomy has no influence upon normal established lactation (Selye and McKeown, '34). Hence, the contention of Mirskaia and Crew ('30) that delayed implantation and prolonged pregnancy are due to the inability of the corpus luteum to maintain lactation and at the same time prepare the uterus for implantation, is without foundation.

Selye ('34) and Selye and McKeown ('34) have shown that the act of suckling reflexly maintains lactation in the rat. Removal of the pituitary gland brings about a cessation of milk secretion. These investigators believe that the act of suckling stimulates the production of prolactin by the hypophysis. Prolactin in turn stimulates and maintains the secretory activity of the mammary glands.

It is possible that with the increased activity of the hypophysis in producing prolactin, there is a corresponding suppression in secretion or release, of the hypophyseal gonadotropic hormones. This might result in a failure of the corpora lutea to develop properly or else bring about a slower development and thus account for the failure of the uterus to respond to the presence of the fertilized eggs. Furthermore, the larger the number of young, the greater would be the suckling stimulus and the greater the suppression of the gonadotropic hormones. This might very well explain the variation in time of implantation between rats suckling small litters and those suckling large numbers.

There is one good argument against this conception. It has been shown by Corner and Warren ('19), Long and Evans ('22), Lyon and Allen ('38) and Leonard and Meyer ('39) that placentomata can be produced in lactating rats from the fourth day to as long as the twenty-fourth day post-partum. In some additional experiments the author has found that if threads are inserted in the uterus on the fourth day after parturition, placentomata may be produced as readily in rats suckling ten young as in those suckling smaller numbers. This would seem to indicate that the uterus is fully capable of responding to the presence of fertilized eggs by the fourth day post-partum even when large numbers of young are being suckled. This, however, is contrary to what actually occurs. It is of course possible, that the mechanical injury produced by threading the uterus might be a much stronger stimulus than the presence of the fertilized ovum. Further experiments are being carried out in an attempt to clarify this point.

The fact that placentomata may be produced as late as the twenty-fourth day post-partum checks well with the fact that implantation may be delayed until 16 or more days after parturition.

Teel ('26) injected anterior hypophyseal fluid into normal pregnant rats and caused a delay in implantation and prolongation of gestation from 2-6 days. In most of his cases the birth mechanism was interfered with. If the injections were not continued too long the young were born alive. The results which Teel obtained really duplicated what occurs naturally when inseminated rats are suckling young from a previous litter. He believed that his results were due to the luteinizing gonadotropic agent in the hypophyseal extracts. It seems strange that one luteinizing factor should cause a delay in implantation whereas another brings about an early implantation. At the time when Teel performed his experiments the extracts containing gonadotropic hormones were necessarily crude. It is possible that some factor in the extracts, other than gonadotropic, may have been responsible for bringing about the delay in implantation which he observed. The author, in

some additional experiments, is attempting to correlate Teel's findings with those recorded here.

CONCLUSIONS

1. The length of the prolonged gestation of inseminated lactating rats is closely correlated with the number of suckling young.

2. The size of the corpora lutea in the ovaries of lactating rats on the twenty-first day of pregnancy is inversely correlated with the number of suckling young.

3. Injection of large doses of corpus luteum hormone (progesterone) may possibly bring about a normal and early implantation of fertilized eggs.

4. Injection of the pregnancy urine gonadotropic factor (Antuitrin-S) brings about normal and early implantation and is successful in maintaining viable fetuses *in utero*. Resorption areas are common.

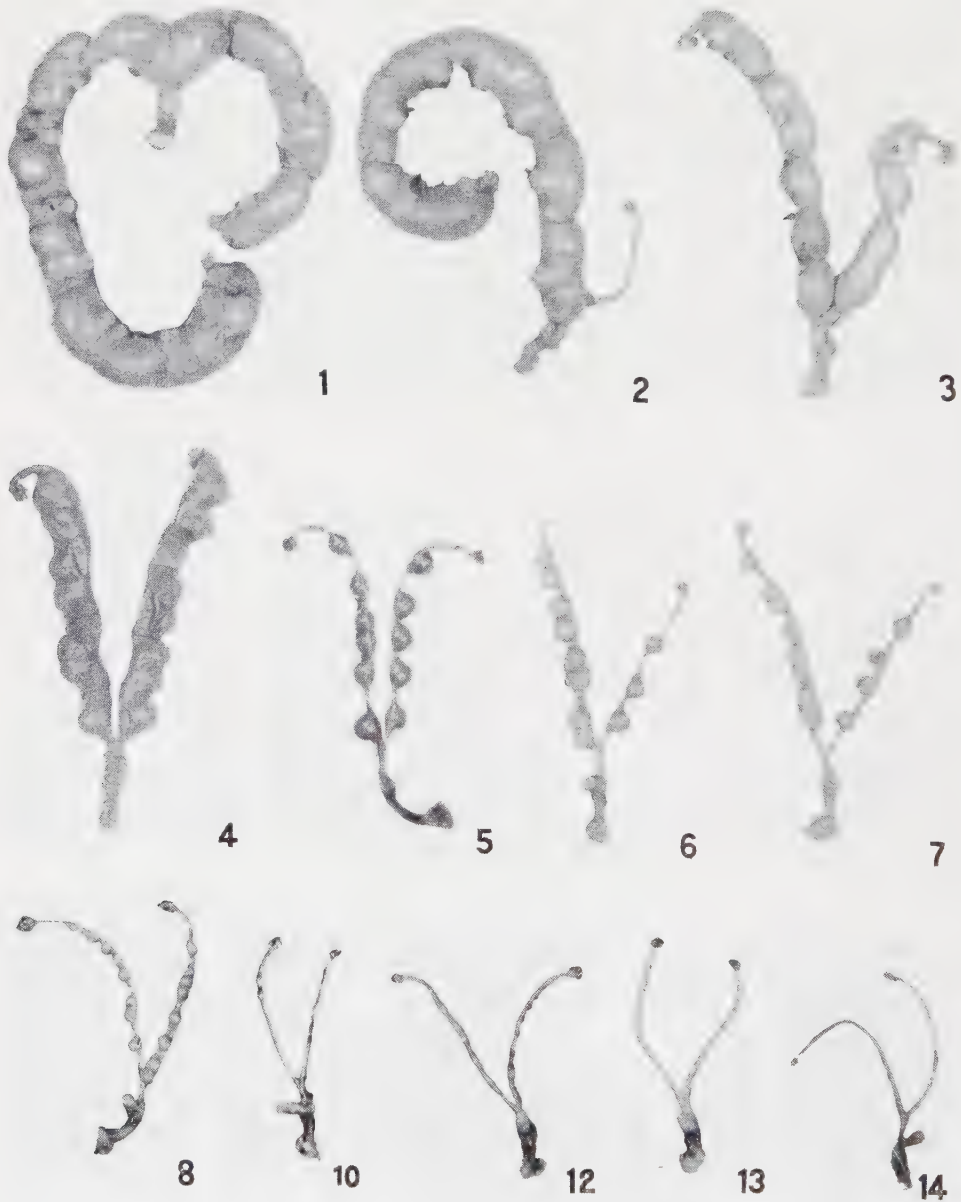
5. Injection of Antuitrin-S supplemented by progesterone is effective in bringing about normal implantation, gestation, delivery and rearing of young in rats suckling large numbers of young.

6. It is suggested that the act of suckling may stimulate the production of prolactin by the hypophysis with a corresponding inhibition of secretion or release, of gonadotropic hormones. The time when ovarian hormones necessary for nidation are released may thus vary with the strength of the suckling stimulus and therefore be dependent upon the number of suckling young.

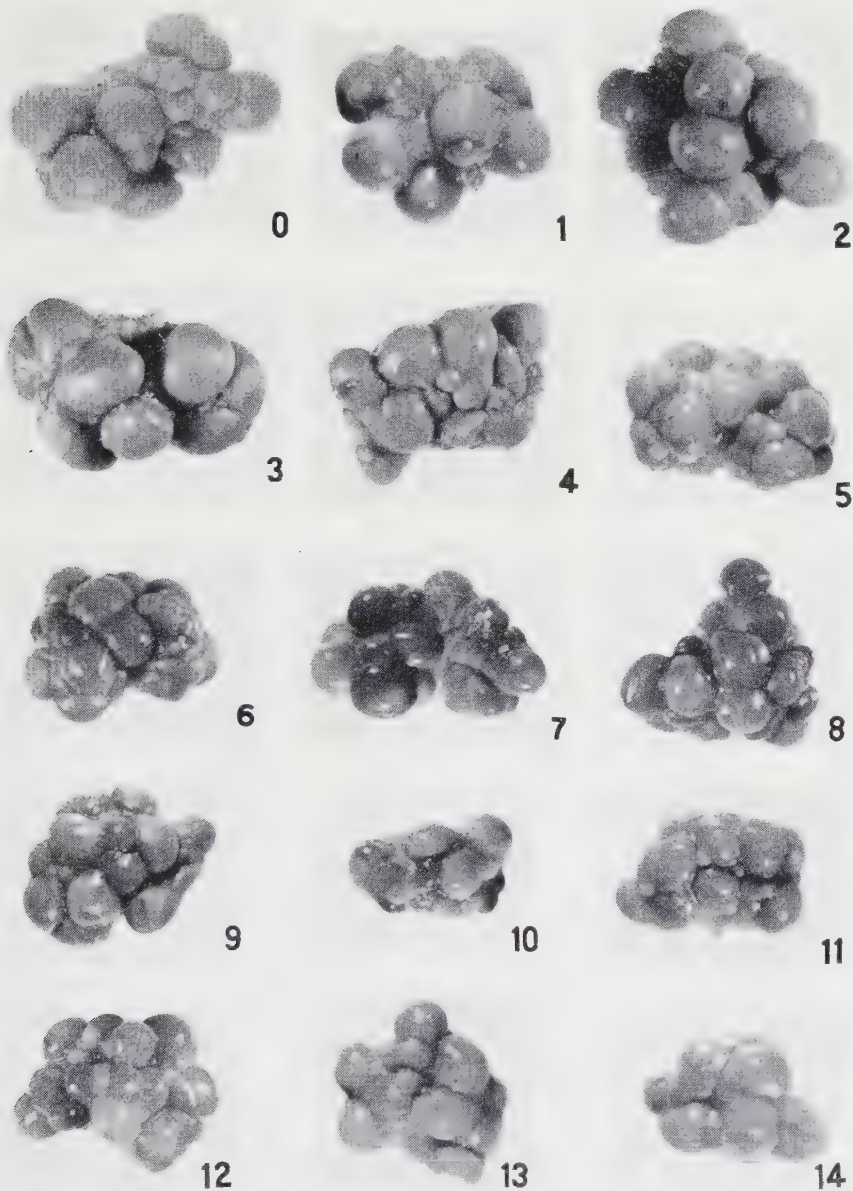
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Showing the variation in size of uteri on the twenty-first day of pregnancy of rats suckling different numbers of young. The number in the lower right hand corner of each figure indicates the number of suckling young from the previous litter.



Ovaries removed from lactating rats on the twenty-first day of pregnancy. The number in the lower right hand corner of each figure indicates the number of suckling young from the previous litter. The progressive diminution in size of the corpora lutea is unmistakable.

PLATE 3

EXPLANATION OF FIGURES

Uteri of experimental lactating rats sacrificed on the twenty-first day of pregnancy. The number in the lower right hand corner of each figure represents the number of suckling young from the previous litter.

A Uterus of rat suckling ten young; injected with 0.2 mg. progesterone on days 3-5-7-9 after insemination.

B Uterus of rat suckling ten young; injected daily with 0.5 mg. progesterone for the first 8 days after insemination.

C Uterus of rat suckling ten young; injected daily for 20 days after insemination with 1.0 mg. progesterone. The embryos are being resorbed.

D Uterus of rat suckling twelve young; injected daily for 20 days after insemination with 3.0 mg. progesterone. Resorption is taking place.

E Uterus of rat suckling ten young; injected daily with 20 rat units of Antuitrin-S for the first 6 days after insemination. Implantation has been delayed.

F Uterus of rat suckling eleven young; injected daily with 20 rat units of Antuitrin-S for the first 8 days after insemination. Note resorption areas.

G Uterus of rat suckling eleven young; injected daily with 20 rat units of Antuitrin-S for 20 days after insemination. Note resorption areas.

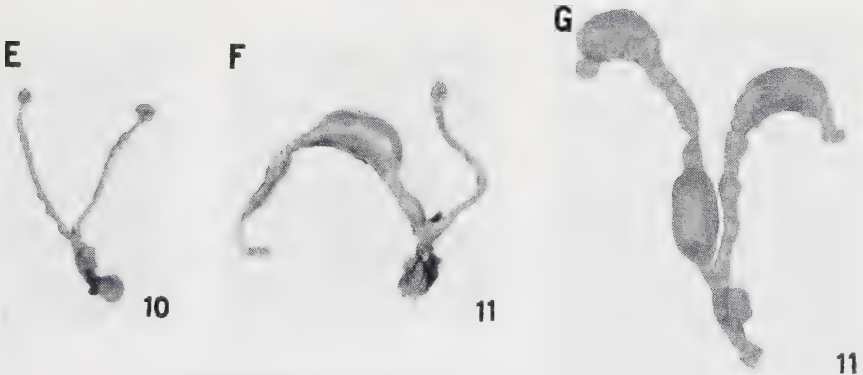
H Uterus of normal control pregnant rat on twenty-first day of gestation. Ten foetuses are present.

I Uterus of rat suckling ten young; injected daily with 20 rat units of Antuitrin-S for the first 8 days after insemination. This was followed by injection of .2 mg. progesterone on days 10-12-14-16-18-20. Eight foetuses are present.

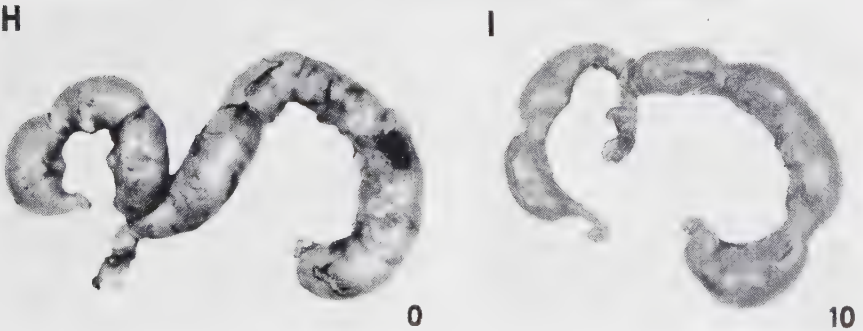
PROGESTERONE



ANTUITRIN-S



ANTUITRIN-S THEN PROGESTERONE



INCOMPLETE POSTERIOR DUPLICATION OF THE BODY (DIPYGUS) IN AN ALBINO RAT

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FOUR FIGURES

The anomalous male here reported appeared in the strain of inbred albino rats that has been maintained at The Wistar Institute since 1909. It belonged in a litter, comprising two males and five females, born December 28, 1938. Litter mates and the forty-seven other offspring of the same parents were normal. Somewhat smaller than other members of the litter at birth, this abnormal male grew very slowly during the suckling period, but otherwise seemed healthy and moved actively about the cage until it was about 45 days old. Then signs of pulmonary suppuration appeared and the abdomen became so distended by the development of several firm, irregular masses that locomotion was difficult. As death seemed imminent, the rat was killed when 56 days old. At this time it had a body weight of 111 gm., the weight of a normal litter brother being 187 gm.

A "double monster" had never been found previously among the inbred albinos, although over 70,000 rats had been born in the strain. It seemed possible, therefore, that this unique individual might be a mutant type, not recorded for the rat, although known to occur in mice where heredity seemingly plays an important role in its development (Danforth, '30). When mature, normal litter mates of this anomalous rat were inbred, as were a number of their descendants through three generations. All of the young, approximately 200 individuals, were normal. This finding indicates that the malformation was

not of germinal origin, but a sporadic case that can be interpreted, in accordance with Stockard's ('21) view, as due to some unfavorable intrauterine condition which caused an arrest or slowing of its developmental rate during a particular period of foetal life.

Head, thorax and anterior extremities of this animal were perfectly formed. Suspended from the anterior abdominal



Fig. 1 Ventral view showing three hindlegs and the imperfect foreleg that developed from the abdominal wall. The mass to the left of the mid-line is part of the fourth hindleg. The bulging lower abdomen is shown.

wall was a narrow, flaccid, tubular structure which terminated in an incompletely developed forefoot (fig. 1). The right hindleg was about twice normal diameter throughout its length and divided longitudinally just above the ankle into two unequal halves each of which carried a complete foot. The lateral one of these feet functioned as the right foot of the animal, while the median one, a left foot, was fully flexed, curved



slightly towards median line of the pair, and fixed in position. The functional left hindleg was entirely normal, but to the left of the anterior mid-line, loosely joined to the pubic region, was a single, complete right hindleg. This was so placed as to drag on its lateral surface, but could be moved into almost



Fig. 2 Dorsal view with the hindlegs and tail in the usual positions when the animal was free.

any position. Whether or not voluntary motion of this member was possible is unknown. In any event, it appeared wasted or under-developed (fig. 2).

The internal structures of the head, neck, and thorax were not unusual except for scattered lobular consolidation of the

lungs. Likewise the liver, spleen, pancreas, stomach, kidneys and adrenals had developed normally. But, about 4 cm. distal to the stomach, a short portion of the small intestine was moderately expanded and beyond this point the entire intestinal tract was duplicated. From the mucous surface of the intestine, the division was less abrupt than indicated by external appearances. Internally the separation began well above the point at which the serous surfaces divided, and

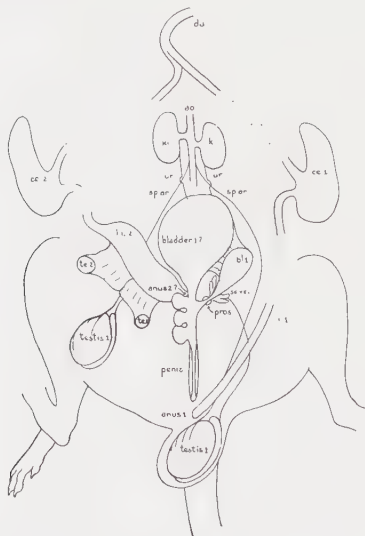


Fig. 3 Diagrammatic sketch of abdominal and pelvic viscera. Outlines of the hindlegs and tail permit orientation, but certain organs were displaced so that anatomical arrangements could be shown. See text for description. (Approximately natural size.)

the tubes were unequal, the left being larger and its ostium wider (fig. 3).

The left branch of the intestine connected with a normal cecum, colon and anus in which the contents were of the usual character. The right branch of the small intestine also joined a cecum and colon, but the cecum and distal half of the large intestine were distended by compacted fecal masses and quantities of inorganic crystalline material.

The expanded terminal right colon and rectum were densely adherent to pelvic surfaces at various points and, in the perineal region, had almost perforated into the subcutis. Dense adhesions also bound the distal end of this segment of the gut to a larger thick-walled bladder-like structure which was placed in the anterior mid-line above the pelvic brim. The right rectum emptied into the neck of this viscus by a small circular opening about 1 mm. in diameter. The mucosa of this bladder, of its opening into the rectum and of the rectum was continuous and intact. The neck of this bladder opened into a thin-walled tube of irregular diameter, the lumen of which expanded into three saccular diverticulae, the last just above the penile urethra, with which the tube was continuous (fig. 3). Surrounding this irregularly expanded channel were dense masses of inflammatory tissue. This bladder-like structure and the sacculated tube contained material similar to that in the expanded colon.

To the left of the large bladder lay the smaller, normal, urinary bladder, attached to it by thin, membranous tissue. Normal ureters, arising in the two normally formed and placed kidneys, entered the neck at the usual position. Rudimentary seminal vesicles and prostate lay dorsal to the neck of the normal bladder which continued as a thin-walled tube (membranous portion of urethra?) to join the channel leading to the penis (fig. 3). The penis was in the normal position, but small, apparently underdeveloped. Bulbourethral glands were not found.

Placed at the left of the tail's base a small scrotal sac contained a normally formed but small testis, from which the ductus deferens passed through the inguinal canal to join the urethra. On the right was found another small testis, placed in a thin-walled subcutaneous sac on the medial surface of the doubled right thigh, nearer the knee than the groin. The duct from this body passed dorsal to the distended right colon and central bladder to the urethra. The spermatic arteries for this pair of gonads were traced to the aorta (fig. 3).

Attached by a short, broad mesentery to the lateral wall of the distended right large intestine were two spherical pale, ivory-white bodies 4-5 mm. in diameter and supplied by blood vessels from the wall of the gut. These were taken to be a second pair of testes.

Opaque material in the blocked half of the intestine and in the central bladder obscured roentgenograms of the pelvic bones, and the bones of the hindlegs were too complex in their arrangement to allow suitable views. Therefore details of



Fig. 4 (A) Bones from appendage attached to abdominal wall. (B) Median aspects of right pair of innominate bones. (C) Ventral aspects of pelvic and adjacent vertebrae. (D) Ventral aspects of left pair of innominate bones.

the skeleton were studied after the body had been cleared and stained under the direction of Dr. E. J. Farris.

The spinal column was not abnormal except for minor variations in the contours of the lateral processes of the vertebral bodies. The pelvic skeleton consisted of a single sacrum to which was attached two pairs of innominate bones (fig. 4, B, C and D). One ileum of each pair seemed fully developed, and articulated with the sacrum. The right pair of innominate bones, sketched in figure 4, B, from the median aspect, formed

a sharply angular and abnormally long symphysis, the apex of which pointed to the mid-line of the animal. In the right half of this pair the pubic bone was short and heavy, the ischium moderately distorted and the ilium approximately normal. In the left half, lack of development of the ilium was particularly notable, while other elements were nearly normal.

Acetabula had formed in the usual position, and the femora articulated normally. These were approximately equal in length, but the median one of the pair measured about half its fellow's diameter. The bones were not joined, although closely placed at the distal ends. However, the tibiae fused at the epiphysis and through the upper third of the shafts, but otherwise had developed completely. The fibulae, on the other hand, did not, as is normal for the rat, fuse with the tibiae, and each was lateral to the companion bone, offering further proof of the opinion that right and left legs made up this complex. Bones of the right functional foot of this pair were complete, but tarsals of the left foot had fused.

Of the left pair of innominate bones (fig. 4, D) the dorsal or left half seemed complete, while the right or ventral half was smaller and the ilium distorted into a horn-shaped structure. The symphysis had not fused, although the pubic bones were joined by a cartilaginous band.

Acetabula also had formed in this side, and bones of the legs were complete, although those of the median, or right leg, were delicate and the shaft of the femur had been fractured and separated in the proximal third.

The tubular structure that was attached to the anterior abdominal wall contained one narrow, elongate bone, the proximal end of which formed a flattened, subterminal bulb. Possibly this was a humerus. Articulating with its distal end was a group of short, thin bones, forming a sort of socket with it and ending in malformed digits (fig. 4, A).

Duplication of parts of the posterior region of the body has been variously classified. The present case corresponds to dipygus of Wilder ('04), whose material was taken mainly

from congenital deformities of man. Usually doubling of the posterior extremities is associated with bifurcation or duplication of the genitalia, the large bowel, and the spine to or above the lumbar segment (Ballantyne, '04; Wilder, '04; Schwalbe, '07).

Only three cases of dipygus have been described in rats, one by Conrow ('17), one by Altman ('26) and the present one. The spine in each of these was single, although deformities of the hindlegs and internal organs were essentially similar to those found in analogous forms of monstrous development described for other species.

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THE EFFECTS OF OVARIAN HORMONES AND SEASONS ON ANOLIS CAROLINENSIS

II. THE GENITAL SYSTEM ¹

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THREE PLATES (THIRTY FIGURES)

INTRODUCTION

Among the gradually increasing studies of hormones in reptiles the following are important in connection with the effects of seasons, sex hormones and gonadectomy upon the genital system and upon behavior. In both sexes of *Phrynosoma solare* (Blount, '29), male *Lacerta serpa* (Herlant, '33), both sexes of *Lacerta agilis* (Regamey, '35), and male *Lacerta agilis* and *L. muralis* (Reiss, '23) the gonads undergo great enlargement during the breeding season and a corresponding decrease in size during the nonbreeding or winter season.

Castration in the males of *Lacerta serpa* (Herlant, '33), *Lacerta agilis* (Regamey, '35), *Lacerta muralis* (Padoa, '33), and *Takydromus tachydromoides* (Takewaki and Fukuda, '35) causes reduction in size of the epididymis; the femoral pores in *Lacerta agilis* and *Lacerta muralis* are reduced in diameter and secretory activity (Regamey, '35 and Padoa, '33). Loss of the brilliant male coloring of *Lacerta agilis* is likewise reported (Regamey, '35). Autoplastic testicular implants effect the restoration of the epididymis to its normal condition (Takewaki and Fukuda, '35). Ovariectomy in *Lacerta agilis* (Regamey, '35) results in reduction of size of the oviducts. Preliminary studies have shown that castration makes no observable difference in the fighting activity of the male *Anolis*

¹ This study was made possible by grants-in-aid from The Elizabeth Thompson Science Fund and The American Academy of Arts and Science.

carolinensis (Evans, '36 a) while ovariectomy is known to have a profound effect upon the fighting response of the female, causing her to respond more quickly and viciously than normal females to an intruder placed on her territory (Evans, '36 b, '37). There are also indications that spaying inhibits considerably the typical mating response of the female to the dewlap display of the mating male in a ratio of 1:3.5 as compared to a ratio of 1:1.18 for the normal female *Anolis* (Evans, '38).

The effects of the female sex hormone or folliculine injected into lizard eggs causes enlargement of the embryonic oviducts in both male and female at time of hatching (Dantchakoff and Kinderis, '37). Folliculin injected into female turtles causes hypertrophy of the oviducts (Kehl, '30). Oestrone injected into immature alligators stimulates the oviducts of both males and females and the ovarian and testicular cortex but has no effect on the medullary portion of the gonad (Forbes, '38). Preliminary work indicates that theelin causes enlargement of the epididymis and atrophy of the testis in mature male *Eumeces laticeps* (Turner, '35).

Hypertrophy of the thyroid follicles of both males and females of *Anolis carolinensis* results under the influence of theelin injections. Especially high thyroid follicular epithelium was found in normal females at the time of ovulation (Evans and Hegre, '38). It is presumed that increased liberation of theelin from the ovaries at time of ovulation is directly or indirectly related to the hypertrophied thyroid in *Anolis*.

This paper describes the effects of seasons, gonadectomy and theelin injections upon the genital system of *Anolis carolinensis*, studying first the results on the male and then on the female.

The animals were secured from New Orleans; were housed, cared for and treated at the Harvard Biological Laboratories. The tissues were prepared and photographs taken (by George Christensen) at Montana State University. The senior author wishes to express appreciation to Dr. A. B. Dawson of Harvard for his friendly guidance and interest.

MATERIALS AND METHODS

Forty-five males were divided into groups of eleven winter controls, sixteen winter injected with theelin, ten summer controls, eight castrates, two of which subsequently received anterior pituitary extracts. Fifty-two females were divided into groups of fifteen winter controls, sixteen winter injected with theelin, eight summer controls, thirteen spayed females of which three received subsequent injections of anterior pituitary extract.

The winter controls of both sexes as well as those which were injected with theelin were sacrificed between November

TABLE 1

NUMBER OF ANIMALS	DATE CASTRATED	DATE KILLED	R.U. SHEEP PITUITARY
Males (average weight, 4.0 gm.)			
2	7/24/34	1/5/35	
2	7/26/34	1/6/35	
2	8/2/34	9/16/34	14
2	3/25/35	4/14/35	
Females (average weight, 2.5 gm.)			
5	2/28/36	6/13/36	
1	7/26/34	9/15/34	14
2	8/9/34	9/13/34	32
2	3/16/35	4/4/35	
3	6/4/34	9/20/35	

27 and December 11, 1935. The summer controls of both sexes were killed on June 12, 1936.

Table 1 gives the number of adult animals of both sexes which were gonadectomized, with the dates when each was operated and sacrificed together with the amounts of anterior sheep pituitary extract subsequently injected into five of these animals. This extract was given at the rate of 2 rat units a day. The animals were killed 24 hours after the last injection.

Aqueous theelin (kindly supplied by Dr. Oliver Kamm of Parke Davis) was injected subcutaneously. The average dosage was 9 rat units administered at the rate of one every 48 hours.

The animals were fed, watered and cared for as described elsewhere (Evans, '36 b). Gonadectomy operations were carried out as aseptically as possible. A separate incision was made through the body wall to remove each gonad. Two or three silk sutures closed the incisions. The animals fed normally 24 hours after the operation and seemed to suffer no ill effects. Autopsy showed no regenerated gonads or any evidence of infection.

Tissues were fixed in either Helly's (7½% formalin) or in Bouin's, cut at 5 μ and stained in Harris' modification of Delafield's hematoxylin.

Only the left gonads and oviducts were weighed. These were dropped, immediately after killing, directly into a weighing vessel containing the killing fluid placed on one pan of the chain balance. There was no observable difference between right and left gonads or oviducts.

RESULTS

1. *Testis, ductus deferens and cloaca*

Table 2 gives the body weight in grams and the weight of the left testis in milligrams for each male of the winter control, or uninjected, series as well as for the series receiving aqueous theelin. Theelin dosage administered to each of the latter series is indicated. Average body weight and the weight of the left testis appear for the summer control males.

Table 3 gives the average dimensions for the width and length of the testis, diameter of the seminiferous tubules, diameter of cloaca, and the degree of spermatogenic activity in the testis of the males of the summer control series and of the winter control and the series injected with theelin. In this table also are the averages for the ductus deferens of the summer and winter series and of the castrate males so far as diameter, height of epithelial cells, number of cells in cross section, and presence or absence of spermatozoa are concerned. Averages for the cloaca in the summer, winter and castrate series with regard to diameter, height of cells and degree of

TABLE 2

Effects of theelin on weight of testes of Anolis carolinensis

NUMBER OF ANIMALS	WINTER CONTROL		SUMMER CONTROL		WINTER THEELIN		
	Body weight	Weight, left testis	Body weight	Weight, left testis	Body weight	Weight, left testis	r.u. theelin
	gm.	mg.	gm.	mg.	gm.	mg.	
1	3.6	9.2	4.3	30.3	3.5	2.0	11.5
2	3.3	5.2	4.0	29.0	3.2	2.8	11.5
3	3.2	13.6	3.9	30.3	3.2	2.9	4.5
4	3.2	11.0	3.6	24.9	3.1	4.0	11.5
5	2.9	5.4	3.5	24.3	2.6	0.9	11.5
6	2.5	8.6	3.4	18.0	2.5	2.0	11.5
7	2.4	9.8	3.4	24.7	2.5	1.0	6.0
8	2.1	7.8	3.3	19.1	2.3	0.6	11.5
9	1.7	3.0	3.2	21.3	2.2	3.6	5.5
10	1.65	1.2	3.2	19.8	2.1	1.0	11.5
11	1.3	1.3			2.0	1.2	11.5
12					1.9	2.2	11.5
13					1.8	2.7	11.5
14					1.5	2.1	4.5
15					1.2	0.6	11.5
16					1.0	1.0	11.5
Av.	2.53	6.46	3.58	24.17	2.28	1.91	9.8

TABLE 3

Effects of theelin and castration on testis, cloaca, and vas deferens of Anolis

	TESTIS				VAS DEFERENS			CLOACA		
	Width in μ	Length in μ	Width of tubule	Spermato- genic activity	Diam. in μ	Height mucosa cells, μ	Cells in cross section	Diam. in μ	Height mucosa cells, μ	Folds of mucosa
Summer control	2900.0	3700.0	152.6	++++	94	16	100	438	30	++++
Winter control	563.6	924.8	146.0	+	55	9	29	360	25	++
Summer castrates					35	10	25	400	20	+
Winter theelin	399.9	668.5	97.5	++	75	16	20	400	25	+++

folding of mucosa also appear in table 3, as well as data concerning gonadectomized males.

Seasonal changes were especially noticeable in the male genital tract. Comparison of summer and winter controls showed that the testes in the former weighed 74% more, were 76% longer and 81% wider than those of the latter. The diameter of the seminiferous tubule was 9% greater in the summer control and the diameter of the ductus deferens was 42% greater. Height of the epithelial cells of the ductus deferens was 44% greater than in the winter controls. The cloaca of summer controls was 18% larger in diameter and the height of the cells of its epithelial lining was 17% greater than in winter controls.

The effect which castration had upon the ductus deferens was to induce atrophy to the extent of a 63% reduction in diameter as compared with summer controls, and a 37% reduction as compared with winter controls. The height of the epithelial cells of the ductus deferens was 38% less in the castrate than in the summer control but slightly greater than in the winter control. Injections of anterior pituitary extract had no effect on the ductus deferens of two castrated males, indicating that the testis is directly responsible for the maintenance of this duct.

The effects of theelin upon the male genital system were marked in spite of the relatively low dosages used. The treated testis weighed 71% less than the winter control testis while in length and width it was 30% less than that of the winter control. The average diameter of the seminiferous tubules was 34% less than in the winter control. Whereas theelin caused retrogression in the testis it had a stimulating effect upon the ductus deferens and cloaca. It was found that the diameters of the treated ductus deferens and cloaca were, respectively, 27% and 10% greater than those of winter controls; the height of the epithelial cells of the treated ductus deferens was 44% greater; the number of epithelial cells in an average cross section was 32% less than in winter controls. This correlates with the fact that the treated ductus deferens

was much more coiled than that of the winter control, the cells being enlarged both in height and width. Hence they would be fewer in number in any given cross section.

2. The ovary, oviduct and cloaca

Table 4 gives the body weight in grams and the weights of the left oviduct and ovary in milligrams for the winter series of the control females, and those injected with theelin and their dosage. It also shows the body weight and the weight of the left oviduct for each of the eight summer controls (killed June 12, 1936) and of five females which were spayed on February 28, 1936 and killed June 13, 1936.

Table 5 gives data on the oviduct and cloaca of summer controls, spayed females, winter controls and the winter series injected with theelin.

Seasonal effects upon oviducts of *Anolis* were similar to those reported by Regamey ('35) for *Lacerta agilis*. The oviduct of the mature summer control was 53% larger by weight than that of the mature winter control. In mature females the thickness of the wall of the lower oviduct from mucosa to serosa was 39% greater in the summer control than in the winter control while the height of the mucosa cells of the lower oviduct was 28% greater than in the winter control. The degree of folding of the mucosa and the thickness of the muscular layer were greater in the summer control.

The cloaca was similarly affected by seasons. The mucosa was highly folded in the summer control and but slightly folded in the winter controls. The muscular layer was much thicker in the summer than in the winter control.

Spaying also affected the oviducts. Those of the summer controls weighed 68% more than those of spayed females although the latter were sacrificed in June within 48 hours of the former. This reduction in weight was 33% less than in the oviducts of five mature winter controls of approximately the same body weight.

The oviduct wall of the spayed female was 45% thinner than the summer control and 10% thinner than in the mature winter

TABLE 4
Effects of theelin on weights of ovaries and oviducts of Anolis carolinensis

Winter							
1. MATURE							
NUM- BER OF ANI- MALS	Body weight	CONTROLS		Body weight	INJECTED		R.U. theelin
		Left ovary	oviduct		Left ovary	oviduct	
	gm.	mg.	mg.	gm.	mg.	mg.	
1	2.5	0.32	9.0	2.7	1.2	11.6	11.5
2	2.5	0.30	5.0	2.5	0.6	11.2	11.0
3	2.4	0.55	0.65	2.5	0.6	10.95	11.0
4	2.3	2.10	9.20	2.5	0.7	9.60	6.0
5	2.0	1.90	8.0	2.5	0.46	4.50	5.5
6				2.5	0.30	9.50	4.5
7				2.5	1.0	12.80	11.0
8				2.2	0.40	4.30	11.0
Av.	2.34	0.93	6.37	2.48	0.65	9.30	8.9
2. IMMATURE							
1	1.8	0.30	0.45	1.9		6.20	10.0
2	1.8	0.23	0.60	1.8	1.40	5.75	11.5
3	1.7	0.42	0.65	1.5	0.20	2.5	11.5
4	1.6	0.70	0.50	1.5	0.40	3.0	11.5
5	1.5		0.40	1.25	0.20	3.2	11.5
6	1.45	0.35	0.30	1.25	0.45	2.95	11.5
7	1.30	0.20	0.30	1.15	0.20	3.10	11.5
8	1.20	0.20	0.30	1.10	0.20	1.60	11.5
9	1.10	0.20	0.30				
10	1.0	0.30	0.30				
Av.	1.44	0.29	0.41	1.43	0.43	3.53	11.3
Summer							
		CONTROLS			SPAYED		
1	2.4		18.3	2.5		4.7	
2	2.9		23.0	2.5		4.8	
3	2.3		16.2	2.2		3.7	
4	2.35		16.1	2.6		3.5	
5	2.1		9.6	2.9		4.9	
6	2.2		5.0				
7	2.5		15.8				
8	2.1		3.7				
Av.	2.3		13.33	2.5		4.32	

control. The height of the epithelial cells of the oviduct of the spayed female was 34% less than the summer control and 10% less than the mature winter control. In the degree of folding of the mucosa of the lower oviduct, there was little difference between the spayed and the winter control females but both were much less folded than the summer control. The muscularis of the lower oviduct was much thicker in the summer control than in the spayed female but in the latter it was thicker than in the winter control.

The cloaca showed less folding of the mucosa in the spayed female than either the summer control or winter control; its

TABLE 5

Effects of theelin and spaying on oviducts and cloaca of mature Anolis carolinensis

	NUM- BER OF ANTI- MALS	OVIDUCT				CLOACA			
		Thickness of oviduct wall, μ	Ht. mucosa cells, μ	Folds of mucosa	Thickness of muscu- laris	Diameter, μ	Ht. mucosa cells, μ	Folds of mucosa	Thickness of muscu- laris
Summer control	8	214	9	++++	++++	500	20	++++	+++
Winter control	5	130.9	6.5	++	+	425	15	++	++
Summer spayed	5	119	6	++	++	495	13	+	+++
Winter theelin	8	150	8	+++	+++	492	17.5	+++	++

muscularis was thicker than the winter control but thinner than the summer control (table 5).

The effects of theelin upon the female in winter are shown in tables 4 and 5. It is difficult to interpret the results obtained in connection with the ovary. In the mature females theelin decreased the average weight of the ovary by 22% while in the immature females it increased the weight by 33%. A similar effect was found in young rats (Freudenberger and Clausen, '37).

An average of 8.9 r.u. of theelin caused an average increase of 32% in the weight of the mature oviduct, while an average

of 11.3 r.u. produced an 88.4% increase in the immature oviduct as compared with mature and immature controls, respectively. In comparing the effects of theelin in the mature and immature females it is necessary to compute dosage administered to each age group in terms of body weight. On this basis the mature females received 3.5 r.u. of theelin per gram of body weight and the immature females received 7.9 r.u. per gram of body weight. Accordingly, the immature oviduct should, theoretically, have registered a 72.2% increase in weight if theelin affected both groups equally. Actually, the immature oviduct showed a 16.2% greater increase in weight than did the mature oviduct, in terms of dosage units per gram of body weight.

Two females received mouse testis implants and their oviducts were greatly atrophied after a period of 6 weeks.

The shell-secreting glands lie in the lower portion of the oviduct beneath the surface of the mucosa and open upon the latter by numerous ducts (Evans, '35). These glands, as well as the cells of the mucosa, showed a remarkable response to changes in seasons, to spaying, to testes implants and to theelin injections as figures 10, 11, 13, 15, 16 and 19 emphasize. The connective tissue and muscular layer of the oviduct likewise showed alterations in response to the same factors. Figures 12 and 14 indicate this so far as theelin injections administered in winter are concerned. Both figures were prepared histologically in identically the same manner.

In the upper oviduct, which secretes albumen, the mucosa layer became highly folded due to the enlargement of the epithelial cells, under the influence of theelin or during the breeding season. Spaying caused atrophy, particularly of the mucosa, as figure 20 indicates.

The cloaca in both sexes was affected by seasons and theelin injections. The folds were thickened with considerable interstitial tissue, capillary plexus and columnar epithelium during the breeding season. Contrast figure 23 with 24 and figure 27 with 28. Theelin increased the height of the epithelial cells. Compare figure 24 with 25 and figure 29 with 30.

DISCUSSION

The evidence, at present, supports the assumption that the entire process of germ cell multiplication and maturation in male *Anolis* is most active during the spring and early summer while the winter stage represents merely a slowed up phase of the process (figs. 1, 2, 4). This differs from the condition in the musk turtle (Risley, '38) and is probably explained by the fact that breeding occurs only in spring and early summer in *Anolis* but in the former in either autumn or spring. The stimulating effect which theelin has upon the ductus deferens of *Anolis* has also been noted in the skink (Turner, '35) and in the rat (Korenchevsky and Dennison, '34; Greene, Burrill and Ivy, '38). A similar effect is reported on the wolffian ducts of newts receiving implants of newt ovaries (Adams, '30).

Whether the deleterious effect of theelin on the testis of *Anolis* can be attributed to its direct action upon the testis or indirectly to the inhibitory effect it had upon the pituitary output of gonadotropic hormones cannot be stated definitely. However, since the effects of theelin upon the ovary were somewhat atropic in the mature females but hypertropic in the immature females, it would seem that theelin had at least some direct effect upon the gonad.

Although in foetal rats theelin caused inhibition of the lower vagina (Greene, Burrill and Ivy, '38), it had uniformly the same effect upon the cloaca of both sexes of *Anolis*, namely the hypertrophy of the epithelial lining.

SUMMARY

Ninety-seven lizards were divided into the following groups: 11 male and 15 female winter controls, 16 males and 16 females injected with theelin in winter, 10 male and 8 female summer controls, 8 male and 13 female castrates of which five had received anterior pituitary extract subsequent to gonadectomy.

The genital system was much reduced in size in winter as compared to early summer. Gonadectomy produced atrophy of oviducts, ductus deferens and cloacae.

Theelin injections caused hypertrophy of the oviducts of females, the ductus deferentes of males and the cloacal epithelium of both sexes. It caused marked atrophy of the testis, slight atrophy of the ovary of mature females but a slight enlargement of ovaries of immature females.

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PLATE 1

EXPLANATION OF FIGURES

Testis and vas deferens of male *Anolis*. All figures $\times 387$. Typical seminiferous tubule of

- 1 Summer, adult control.
- 2 Winter, adult control.
- 3 Winter, adult, $11\frac{1}{2}$ r.u. of theelin.
- 4 Winter, immature control.
- 5 Winter, immature, $11\frac{1}{2}$ r.u. of theelin.

Vas deferens

- 6 Summer, adult control. Shows part of two loops of vas deferens.
- 7 Winter, adult control.
- 8 Winter, adult, $11\frac{1}{2}$ r.u. of theelin.
- 9 Summer, adult castrate.

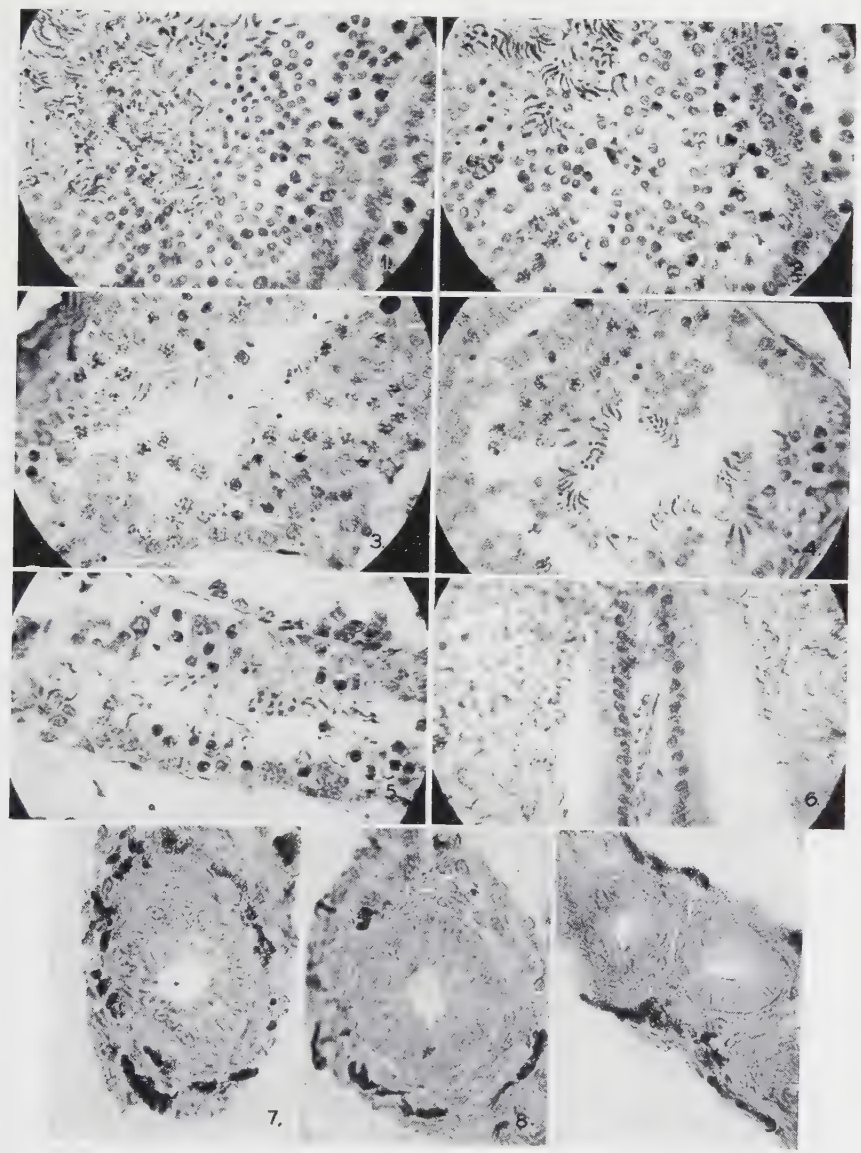


PLATE 2

EXPLANATION OF FIGURES

Oviducts of female *Anolis*. All are $\times 140$ except figures 12 and 14 which are $\times 267$.

Lower oviduct.

- 10 Summer, control, ovulating.
- 11 Winter, control.
- 12 Winter, control.
- 13 Winter, theelin, 11.5 r.u.
- 14 Winter, theelin, 11.5 r.u. Note black-stained connective tissue in contrast to figure 3.
- 15 Summer, spayed.
- 16 Summer, one mouse testis implanted 6 weeks before being killed.
- 17 Summer, control, upper oviduct.
- 18 Autumn, spayed, 32 r.u. sheep pituitary, lower oviduct.
- 19 Autumn, two mouse testes implanted, 32 r.u. sheep pituitary, lower oviduct.
- 20 Summer, spayed, upper oviduct. The mucosal surfaces are shown adhered, with no lumen.
- 21 Winter, control. Lips of ostium (upper end of oviduct). Note cilia on upper or inner surface.
- 22 Winter, theelin, 11.5 r.u. Lips of ostium. Note cilia on lower or inner surface.

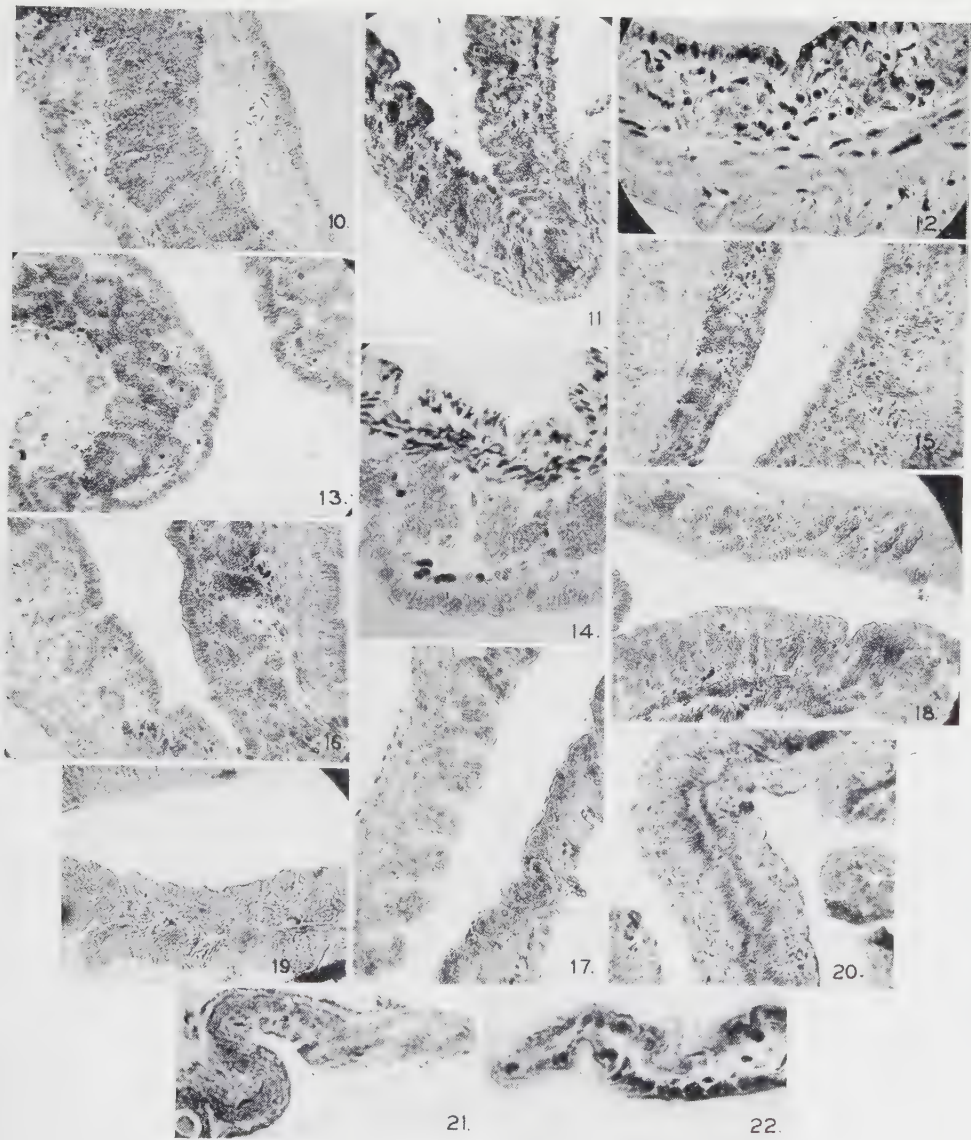


PLATE 3

EXPLANATION OF FIGURES

Cloaca of male and female *Anolis*. Figures 23 to 25, $\times 387$; figures 26 to 28, $\times 140$; figures 29 and 30, $\times 267$.

23 Summer. Male control. Only part of one fold of the cloaca is shown. Note large submucosa region with prominent capillaries.

24 Winter, male, control.

25 Winter, male, theelin, 11.5 r.u.

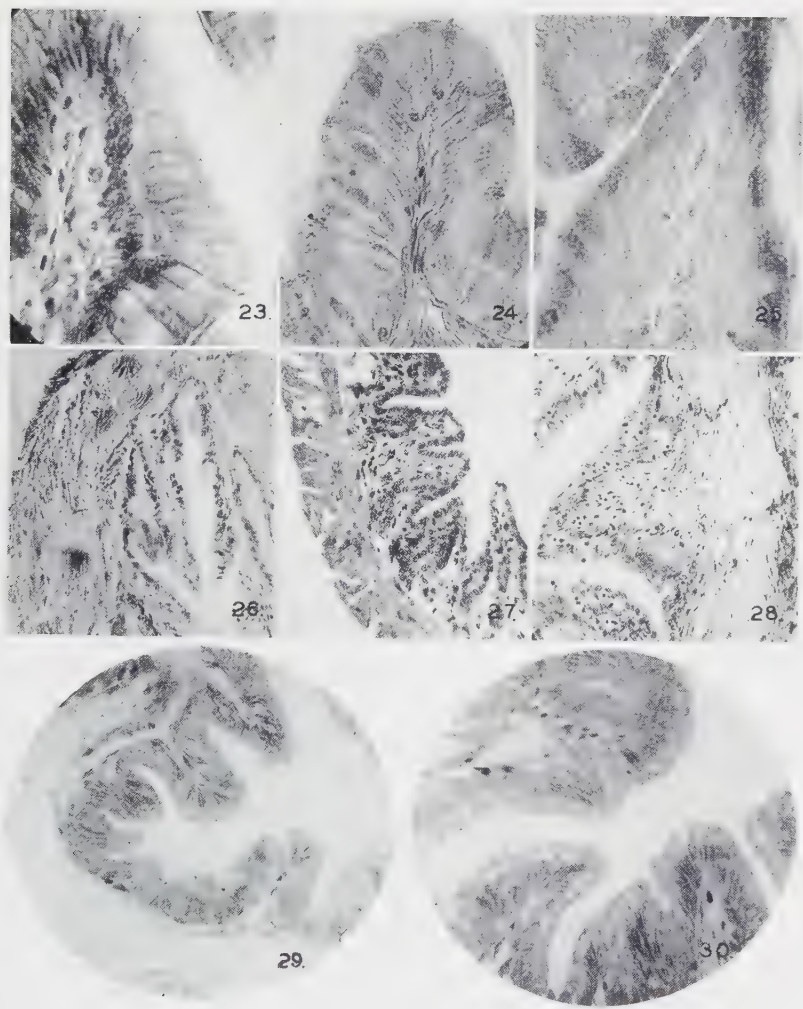
26 Winter, female, theelin, 11.5 r.u.

27 Winter, female, control. Note the thin muscular and connective tissue wall as compared to figure 26.

28 Summer, female, control. Note relatively large mucosal folds as compared to figures 26 and 27.

29 Winter, female, control. Detail of mucosa.

30 Winter, female, theelin, 11.5 r.u. Detail of mucosa.



THE DEVELOPMENT OF THE MEGAKARYOCYTE IN ADULT MICE ¹

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THREE TEXT FIGURES AND TWO PLATES (NINETEEN FIGURES)

Howell (1890) gave the name megakaryocyte to the large cell with a lobate nucleus found in the bone marrow and in the blood forming organs of embryos. Jordan ('25) shifted the term megakaryocyte to a mononuclear cell and called the lobulated nuclear form a polymorphokaryocyte, but this change in terminology has not been generally accepted.

The derivation of this cell has been variously described. Howell (1890) believed that megakaryocytes resulted from the growth of small lymphoid cells, and Flemming (quoted by Pugliese, 1897) described them as abnormally developed lymphoid cells. Pappenheim (1898) derived them from his lymphoidocyte, while Blumenthal ('08), Duesberg and Tommassi (cited by Gruner, '13) ascribed their origin to stellate connective tissue cells. Dickson ('08) was undecided as to whether they arose from the myeloid series or from cells of the connective tissue type. Heidenhain ('11) called them giant leukocytes. Sanfelice (quoted by Gruner, '13) reported that megakaryocytes were produced by the fusion of several leukocytes. Jordan ('18, '20) referred formation to the hypertrophy of hemoblasts. Fontana ('29) derived them from polyblastic histiocytic elements and described certain stages in their development as polykaryocytes and premegakaryocytes. Lapidari ('29) concluded from his experiments that primordial bone marrow cells (hemocytoblasts) were the stem cells, and Rothermel ('30) stated that free lymphoid cells,

¹ Supported by a grant for the study of leukemia from the Carnegie Corporation.

also referred to as hemocytoblasts, could develop into megakaryocytes. Downey, Palmer and Powell ('30) presented evidence for derivation either directly from the reticular stellate cells of the liver sinusoids or from myeloblasts. Storti ('32) suggested the irregular mitosis or amitosis of megaloblastic basophiles as a probable origin. Bloom ('32) found that under certain circumstances fixed non-storing cells of the hepatic sinuses of rabbits transformed directly into megakaryocytes, but pointed out that this was probably exceptional. Kingsley ('34, '37) reported that megakaryocytes developed from hemocytoblasts which differed from other stem cells only by the presence of specific granules. He described nuclear changes progressing from a spherical form to a kidney-shaped and horse-shoe-shaped structure to the stage where projections developed resulting in a complex nucleus. Howell and Donahue ('37) stated that development was from myeloblastic cells.

From this brief survey of the literature it is evident that the reports on the origin and development of the megakaryocyte are not in agreement. Tissues from man, rabbit, cat, pig, mongoose and rat have been used in the various studies, but to the best of our knowledge there are no comparable data concerning the cell type in mice. This paper will present an interpretation of the histogenesis and development of the megakaryocyte in the adult mouse.

OBSERVATIONS

Tissues were taken from normal mice of the four inbred strains C58, Bagg-albino, 89, and Storrs-Little. In addition, pathological material was obtained from cases of spontaneous and transplanted leukemias in strains C58 and Bagg-albino, and also from three cases of extensive extramedullary myelopoiesis in C58 mice. Since no apparent differences except in distribution were found between the cells in normal and pathological material they will not be considered separately. Sections, imprints and dry smears were examined after various fixation and stains had been used to emphasize particular cell and tissue structures.

In the bone marrow of normal mice megakaryocytes are scattered at random, while in the spleen they seem to be concentrated in the subcapsular and peritrabecular red pulp, although individual cells are present in other portions of the red pulp. Occasionally they are found in the medullary cords of normal lymph nodes. We have not seen them in the lungs, liver or circulation of normal adult mice. In the leukemias, more particularly in the spontaneous types, megakaryocytes have been observed in greater or smaller numbers in infiltrated areas in lung, liver, kidney, bone marrow, spleen, lymph nodes, thymus, muscle and in the circulation. Megakaryocytes in the lesions was one of the constant characteristics of transplantable leukemia line R, which provided a large amount of easily accessible material. Isolated regions of extramedullary formation of megakaryocytes were seen at times in lymph nodes but frequently in liver and were of great aid because of their circumscribed and therefore relatively unconfused picture.

Our method of approach to the manner of development of the characteristic mature megakaryocyte has been purely morphological; namely an arrangement of related cell types into a series leading up to the easily recognizable stages.

In all regions of cytopoiesis in myeloid and lymphoid tissues we have found a morphological cell type common to both. It is a large lymphoid cell with basophilic cytoplasm and a large vesicular nucleus usually containing one large plasmasome nucleolus. This cell seems to originate from undifferentiated reticulum cells and is apparently the last developmental stage that the megakaryocyte has in common with other cells of both normal and extramedullary myeloid and lymphoid tissues. We agree with Rothermel ('30) and others that it should be referred to as a hemocytoblast, being well aware of Downey's statement ('30) that, "Whether this stem-cell is regarded as a myeloblast, lymphocyte, or connective tissue cell depends on the particular theory of the origin of extramedullary myeloid tissue that is favored." An example of this cell type is shown in figure 2. Within the nucleus of certain of these

All photographs (except where noted) are from strain C58 mouse tissues, fixed in Helly, sectioned at 8 micra, and stained in hematoxylin and eosin azur II. Magnification x 1000.

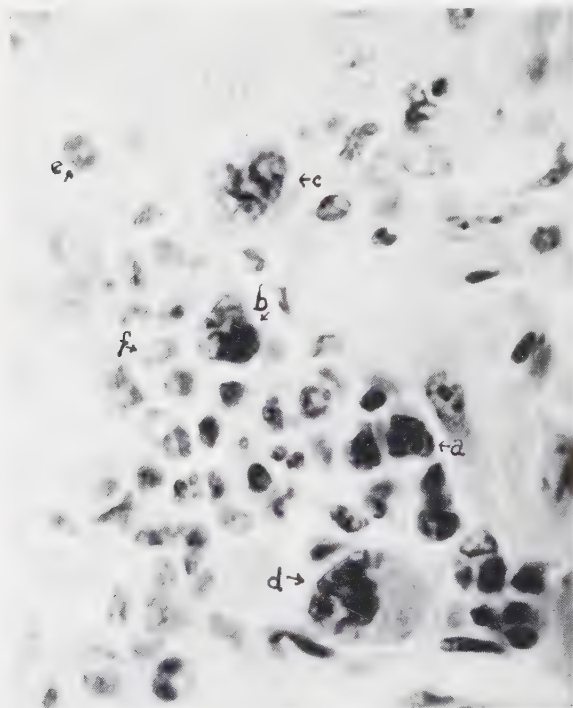


Fig. 1 Section through an area of periportal myelopoiesis in the liver showing megakaryocytes in various stages of nuclear differentiation from the youngest type (a), through the ring forms (b and c) to the oldest type (d). Compare b and c with e and f which are ring forms of the myeloid series.

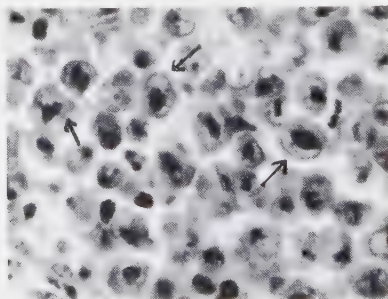


Fig. 2 Hemocytoblasts in normal adult lymph node.

cells, in normal and extramedullary myeloid tissues, there appears a small perforation which, by comparison within a series of such cells, is seen to enlarge to form the "ringkern" of the myeloid series described by Petri ('33) and others as typical in the mouse. The megakaryocyte "ringkern" is larger than the proleukocyte (plate 1) but both go through the same nuclear stages up to the time of incomplete mitosis as described below. In some cases the nuclear ring may be seen to twist upon itself, while in others a break sometimes seems to occur at one point in the circle. In these large cells prophase changes occur in the nucleus and multipolar chromosome multiplication takes place which is not so complicated as in the larger megakaryocytes (plate 2). From this stage onward a series of definable megakaryocytes with increasing nuclear complexity and with an increase in the amount of cytoplasm may be seen (plate 1). In some pathological conditions there is evidence of a series within a single lesion, as for example, in the periportal lesion in a liver, a section of which is shown in figure 1. The large megakaryocyte of the adult mouse has a polymorphous nucleus with many lobulations all connected to form one irregular mass which, in counterstained Feulgen preparations, shows varying numbers of plasmasome nucleoli.

The nuclear changes that periodically occur in cells of this type are of particular interest. We have never seen evidence of multiplication of megakaryocytes, either mitotic or amitotic. The prophase condensation of the chromosomes, arranged on the inner wall of the various lobules, is followed by the formation of a sometimes highly complicated multipolar metaphase. In no case have we seen figures that would indicate that there is a true telophase with the formation of daughter nuclei which subsequently fuse to form the typical interphase nucleus. It seems more probable that after multiplication of the chromosomes a nuclear membrane forms about the confused chromatin mass. The extremely complicated multipolarity of the spindles is evident in the confusion of planes at metaphase, as indicated in plate 2. Although certain ob-

servers have stated that spindle fibers are not formed (Jordan, '20), they are readily demonstrated in the mouse by appropriate cytological methods (plate 2).

The cytoplasm of the megakaryocyte in air dried imprints stained with Wright-Giemsa is faintly basophilic, with azure granules which give the cytoplasm a flocculent appearance. We were unable to show specific granulation in the various stages of the megakaryocyte by the Kingsley method. Supravital staining with Janus green shows many small mitochondria. No peroxidase reaction could be demonstrated by the use of the Sato method, although granular leukocytes in the same field and those engulfed by the megakaryocyte gave a positive reaction (fig. 3). The repeated failure to obtain positive oxydase or peroxidase reactions in the megakaryocyte (Richter, '25; Knoll, '27; Barta, '32; Smith et al., '38) refutes the interpretation by Jackson ('04) and Gruner ('13) that the cell is an abnormally large myelocyte. However, because of the peculiar formation of a ring-shaped nucleus in the early developmental stages of both granular leukocytes and megakaryocytes in the mouse, there seems to be no doubt concerning their close relationship.

The function of this cell is not well-known. It is assumed by many authors that the platelets of the circulating blood originate through budding of the cytoplasm of megakaryocytes (Wright, '10, and others). Petri ('26) and Cicinnati ('28), however, obtained experimental results which they interpreted as showing no relationship between the platelets and megakaryocytes. Most investigators believe that the megakaryocyte is not phagocytic. Kingsley ('34) suggested that the apparent phagocytosis was "only a two-dimensional appearance of cells pushed into bays of megakaryocyte protoplasm." In sections and imprints used in the present study it was usual to see cells and amorphous cellular debris within the cytoplasm of some megakaryocytes, giving the appearance of true phagocytosis. Typical cases are shown in plate 2. Downey et al. ('30) saw suggestive cases of this sort in their

material but remained undecided about the phagocytic power of the megakaryocyte in humans.

To summarize our interpretation of the formation of the megakaryocyte in adult mouse tissues, it seems that after the hemocytoblast begins to differentiate in the megakaryocyte direction a series of intranuclear changes occurs. These

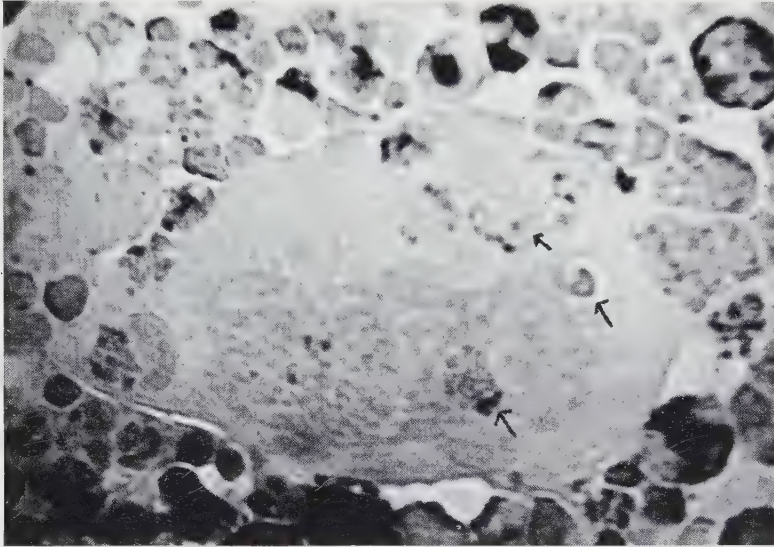


Fig. 3 Sato preparation of normal bone marrow imprint showing a negative peroxydase reaction in the megakaryocyte and a positive reaction in surrounding myeloid cells. Arrows point to inclusions (?).

changes, involving chromosome multiplication with no cellular division, produce a giant polyploid cell with a polymorphous nucleus. The same pattern of origin was observed in bone marrow, spleen and extramedullary myelopoiesis in the liver. The megakaryocyte is incapable of self perpetuation. But, from our morphological studies it appears that regardless of its unusual nuclear history the megakaryocyte should be considered as a definite entity with a definite pattern of development, and not as an abnormal variant. Its function remains ill defined.

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PLATE 1

EXPLANATION OF FIGURES

4 Region of myelopoiesis in spleen showing hemocytoblast (hebl), mitosis (mit), ringkern form of a proleucocyte (prolk), and a young megakaryocyte (ymgk).

5 Section of spleen showing megakaryocyte with ring-shaped nucleus. Compare with small ring form of polymorphonuclear leucocyte to the left.

6 Section of spleen showing megakaryocyte (mgk) in which the nucleus is twisted upon itself and is lobulated. Below is a megakaryocyte with a large nuclear perforation.

7, 8, 9, 10, 11 Sections of spleen (except for 7 which is from liver) showing variations in the ring-shaped nucleus and beginning lobulation.

12 Section of spleen showing inclusions (?) of leucocytes within the cytoplasm of a mature megakaryocyte.

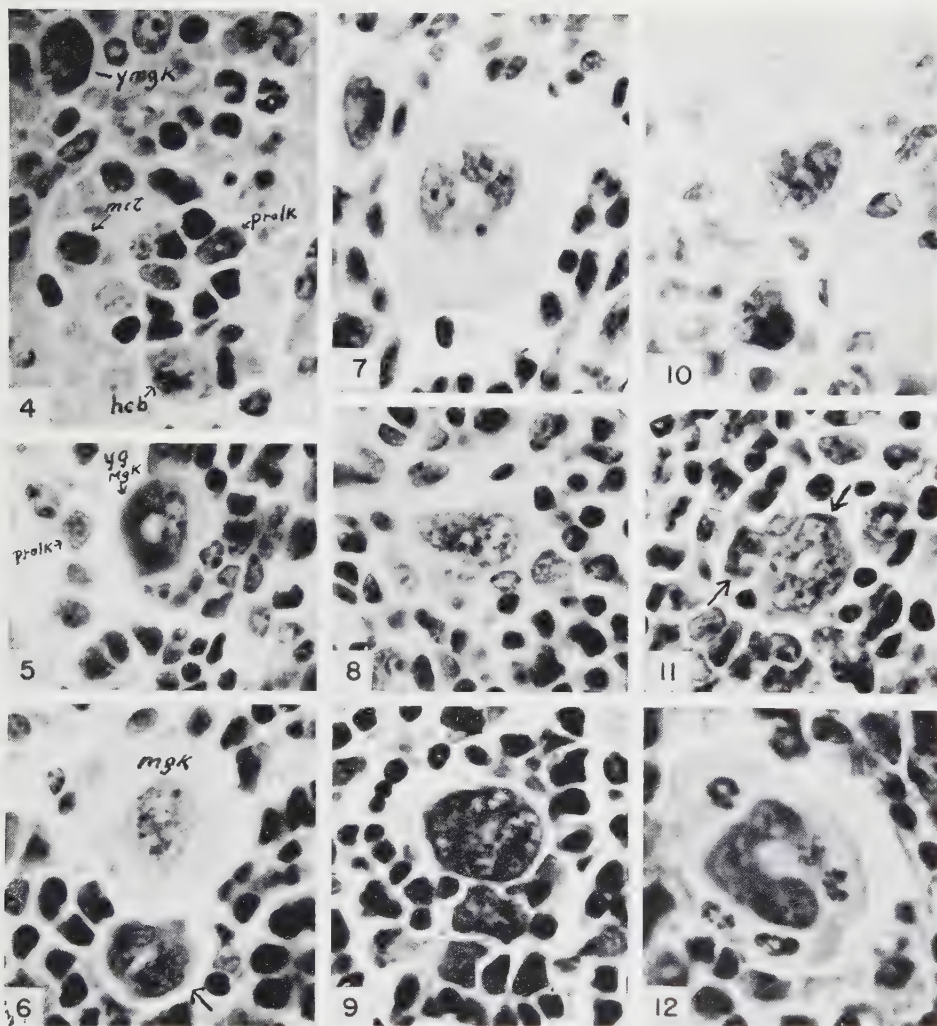


PLATE 2

EXPLANATION OF FIGURES

13 Imprint of normal bone marrow, Wright-Giemsa stain. Mature megakaryocyte showing lobulated and convoluted nucleus, and azurophilic granules in the cytoplasm.

14 Living megakaryocyte in culture medium. Convolutions of nucleus apparent.

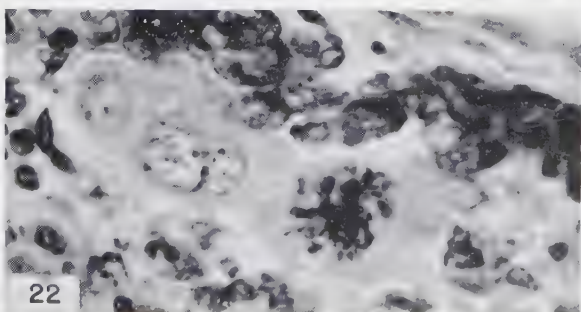
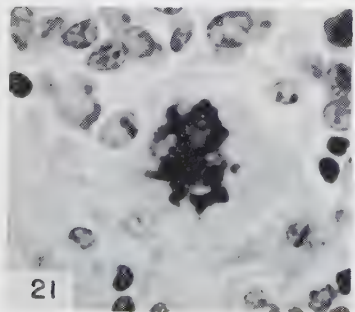
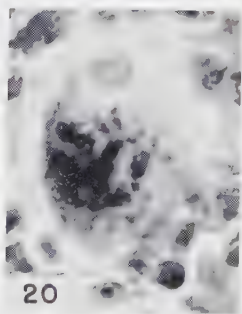
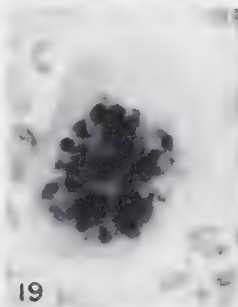
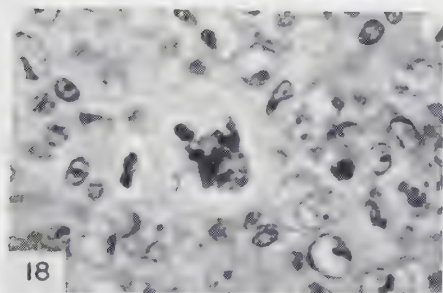
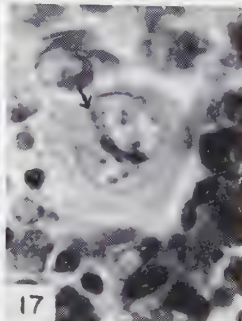
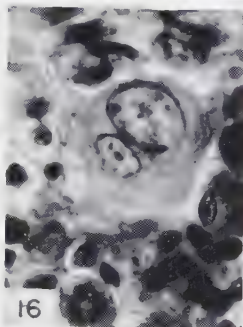
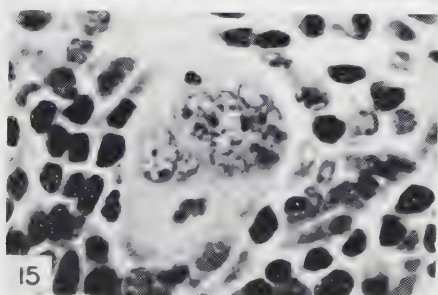
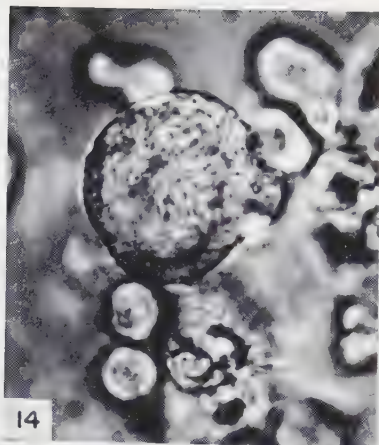
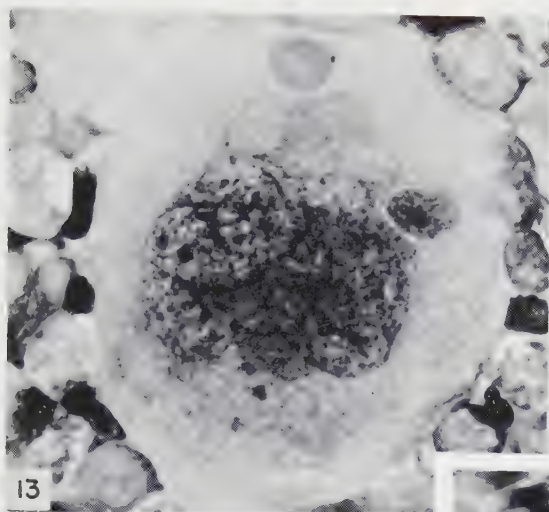
15 Section of spleen showing megakaryocyte with an extremely complicated nucleus, and apparent ingestion of cellular debris.

16 and 17 Section of spleen showing the same megakaryocyte in lower and upper focus to demonstrate that what appears to be two nuclei in 16 is really a single lobulated structure. Arrow in 17 marks the band which joins the two parts.

18 Section of lymph node, Flemming fixation and stain. Chromosome multiplication in megakaryocyte showing well-defined spindles in a quadripolar figure. Note the bipolar division to the left.

19 and 20 Sections of lymph nodes. } Showing the complex multipolarity of

21 and 22 Sections of spleen. } large megakaryocytes.



THE ORIGIN AND SIGNIFICANCE OF THE MEGAKARYOCYTES OF THE LUNGS

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ONE PLATE (FIFTEEN FIGURES)

The recent claim of Howell and Donahue ('37) that the lung is an important organ of megakaryocyte development and platelet production has reopened the question of the origin and significance of the intracapillary giant-cell nuclei of the lung and of the occasional apparently active small "megakaryocytes" which may appear in that organ. These nuclei and cells have been known and described by various authors since attention was first directed to them by Aschoff in 1893. The literature is fully reviewed by Howell and Donahue. The prevailing interpretation regarded these cells as blood-stream emigrants from the bone marrow. Howell and Donahue incline to the view of local origin from pulmonary myeloblasts, and conclude that "the lungs constitute the main source of platelet production." This conclusion is based chiefly on results of experiments with cats, but is extended also to dogs and tentatively to other mammals and man. It calls for a reversal of the previously widely held and well established view that the bone marrow constitutes practically the exclusive source of platelet production in the adult. It may be regarded as a challenge to histologists to prove that the megakaryocytes of the lungs are solely of extraneous origin and that platelet production in the lungs is of only minor functional importance and only incidental to megakaryocyte activity in the bone marrow.

This investigation was limited to the cat. Observations were made on three kittens and three adults. In the preparation of the microscopical material the technic of Howell and Donahue

was followed in detail. On finding that the same varieties of giant nuclei and giant cells that occurred in the alveolar capillaries appeared also in the microscopical sections in the main branches of the pulmonary artery, blood smears were made direct from the pulmonary aorta, the right ventricle and the inferior vena cava. Occasional smears contained a megakaryocyte in some stage of degeneration.

DESCRIPTION

The prevailing varieties of megakaryocytes in the lungs are represented in figures 1 and 2. Practically naked nuclei almost completely fill an alveolar capillary. The nuclei are variously distorted, generally elongated, to fit the caliber of the capillary lumen (figs. 1 and 2). With Wright's stain they have a blue color of varying shade. Cytologically, they vary from a condition showing coarse nuclear rods and threads to one of almost homogeneous structure. These variations signify degrees of degeneration. Where a nucleus gets caught at the point of branching of a capillary it may become bifurcated, a nuclear limb extending into each capillary branch (fig. 2). Such naked nuclei may retain apical fragments of granular cytoplasm (figs. 1 and 2). The granules of these cytoplasmic remnants have the characteristic reddish purple color of the granules of blood platelets. Later stages of nuclear dissolution are represented by a succession of two to four smaller spheroidal nuclei in close series in a capillary. Figure 3 illustrates such a case. Here the smallest nuclear mass consists of a vesicular core and a narrow deeper staining rim. This condition signifies a subterminal stage of nuclear resorption. The terminal granular body of this same series has the character of a huge plastid (platelet) and represents presumably a granular cytoplasmic remnant of the original single polymorphous nucleus of an earlier more complete megakaryocyte. Similar plastids occur fairly frequently in the alveolar capillaries (fig. 4).

Next in order of frequency occur small intracapillary megakaryocytes with variable larger masses of granular cytoplasm. Figure 5 shows an intracapillary polymorphous nucleus with

a polar cap of granular cytoplasm and three typical closely adjacent platelets. Such polar caps may possibly in some cases represent adherent groups of fused platelets. Figure 6 illustrates a condition similar to that of figure 5; here the nucleus appears more nearly normal and there is a considerably larger mass of granular cytoplasm. In figure 7 the amount of granular cytoplasm is still more extensive.

The most interesting and significant conditions are those where similar megakaryocytes and giant nuclei occur in the several subdivisions of the pulmonary artery. Figure 8 shows a typical basket nucleus in a precapillary arteriole. The nucleus is practically naked except for small adherent remnants of granular cytoplasm. Figure 9 shows a similar "cell" in the lumen of an arteriole. The lumen of a large branch of the pulmonary artery, accompanying a secondary bronchus, contained the cells shown in figures 10 to 14.

The nucleus of cell, figure 10, is approaching a homogeneous condition. It stains only lightly, and presents various features of degeneration. Figures 11 and 12 illustrate two successive sections at $7\ \mu$ of the same cell. The nucleus of this megakaryocyte appears approximately normal. The cytoplasm, however, is restricted to a relatively small area of the nuclear surface. It projects as long stout pseudopods, filled with characteristic platelet granules. Figure 13 shows a typical "basket" nucleus. Only two small agranular remnants of the original cytosome remain attached to the nucleus. This nucleus would have been carried distad in the lung, and lodged in a precapillary arteriole would have given essentially the appearance of figure 8. Figure 14 is of an approximately normal megakaryocyte with an enormously long slender granular pseudopod. These cells of the pulmonary artery would necessarily become lodged ultimately in an alveolar capillary. The long pseudopods, if they remained intact, would become stretched along the lumen of capillaries, giving an appearance essentially like that illustrated by Howell and Donahue. Segmentation of these pseudopods would produce platelets identical with those produced in the bone marrow.

Figure 15 represents a megakaryocyte from a stained spread of blood taken directly from the pulmonary artery after death by chloroform. It is a normal giant cell, with basket nucleus and a granular pseudopod in preparation for segmentation into three blood platelets. This cell is essentially identical with megakaryocytes in stained smears of bone marrow. This particular blood smear (20 by 30 mm.) contained four giant cells. Spreads of blood taken from the inferior vena cava just above the diaphragm showed similar giant cells. At least one giant cell or nucleus could be found in most of the spreads of an area of 20 by 50 mm. Both in the blood smears and in the marrow spreads these giant cells generally have a peculiar appearance. Only in the pseudopods do the granules have the characteristic reddish purple color of isolated platelets. In the main mass of the cytosome the granules have a deep blue color. In addition, there occurs a small variable number of small oval bodies, presumably the result of fusion of small granules in the trauma incident to the spreading of the blood or marrow.

Only in the pseudopods and peripheral regions of the cytosome where they are grouped do the granules approach the size of those of the separate platelets. The granules of the main central portion of the megakaryocytes are of smaller size. The granules presumably undergo a process of maturation, suggested superficially by growth, preparatory to platelet liberation. In the sections of the lungs the granules of practically all of the megakaryocytes and of the giant platelets are of definitive size.

The nucleus of the megakaryocytes in these blood and marrow smears is only vaguely outlined. Structural details are not evident; they are masked by the large overlying mass of cytoplasm. The cytosome proper has a blue color of variable shade. An occasional megakaryocyte may be spread out fairly thin, in which case the finer granules of the thinner regions of the cell appear reddish purple as in the pseudopods. Such cells and the naked nuclei in the blood of the right ventricle and the pulmonary aorta must have come from the bone marrow; and they would naturally be carried ultimately to the capillary

mesh of the pulmonary alveoli. Meanwhile they may produce platelets, and ultimately the naked nucleus will undergo dissolution in an alveolar capillary. The evidence seems conclusive that the megakaryocytes of the lungs are of extraneous origin.

This technic reveals also large numbers of mast cells, more abundant in the perivascular, peribronchial and subpleural regions. These cells are in general relatively small, of irregular stellate form, with small central oval nucleus. Adjacent to capillaries these cells may protrude pseudopods into the vascular lumen. The granular pseudopods may constrict off apical plastids, very similar to platelets. If mast cells produce heparin, as claimed by Holmgren and Wilander ('37), the wide distribution of these cells in the lungs would explain the relatively rich content of heparin in this organ.

DISCUSSION

The conclusions of Howell and Donahue that the megakaryocytes of the lungs are "formed or developed" locally from myeloblasts and that "the lungs constitute the main source of platelet production" are based largely on their experimental results. Histological appearances in the lungs are interpreted as supporting these conclusions. These investigators made comparative counts of platelets in companion arteries and veins. With few exceptions the arterial blood showed a slightly larger number of platelets than the venous blood. Among a total of forty-four observations, thirty-four gave a higher count (approximately 10%) of platelets in the artery, eight gave an equal count, two showed a higher count in the vein.

The difference in platelet count as between arterial and venous blood is not striking; it might not actually have functional significance. The small difference in number of platelets in arterial and venous blood might simply be the result of a difference in the degree of pressure in these two bloods. The lower pressure in the blood of the veins allows for a greater degree of adherence of larger groups of cohering platelets to

the vessel wall at the time blood is collected for examination as compared with conditions in arterial blood.

Rather more difficult of explanation is the "more or less steady increase" in the ratio of platelets to red blood corpuscles in successive effluents when the lung was perfused through the heart with a mixture of saline phosphate and albumin, a "platelet preserving solution." Before perfusion the ratio of platelets to red blood corpuscles was approximately 1 to 16. At the end of 30 minutes, after five perfusions, there were approximately from two to eight platelets to each red blood corpuscle. Howell and Donahue interpret these results of their experiments as indicating "some kind of a supply of platelets in the lungs."

Examination of my sections of the lungs of the cat appear to give the explanation for this increase in the ratio of platelets to red blood corpuscles at later intervals during continuous perfusion. It must be recalled that these experimental animals were bled and the blood first washed out with saline. On bleeding from the aorta the blood slowly leaves the lungs through the veins to the left atrium. The slow flow of the blood provides favorable conditions for platelets to clump and adhere to the walls of the smaller branches of the pulmonary veins. My microscopical sections show this very striking condition: In certain areas the capillaries contain large numbers of platelets, and the postcapillary veins and venules of such areas are in some cases completely filled with platelets; barely a single red blood corpuscle and very few leukocytes are included among these coherent masses of platelets. When the animal is bled conditions in the lungs are such as to allow the red blood corpuscles to pass through the smaller venous vessels while the platelets are retained. Judging from appearances in microscopical sections one may conclude that the steady increase in the ratio of platelets to red blood corpuscles with continuous perfusion is the combined result of an enormously reduced content of red blood corpuscles and a washing out of the coherent clumps of platelets in the capillaries and smaller veins.

As regards the increase in the number of pulmonary megakaryocytes in the experiments of Howell and Donahue where the number of circulating platelets was first reduced by "repeated bleeding in small lots and reinjection of blood after defibrination" and sections of the lung examined at the beginning of platelet regeneration after an interval of from 3 to 5 days, it does not necessarily follow that the relatively larger number of giant cells in the lungs is the result of local production. Since "megakaryocytes" (mostly giant nuclei with minimal amounts of granular cytoplasm) of bone marrow origin pass normally through the pulmonary aorta to the lungs, it may be assumed that when megakaryocyte increase is experimentally stimulated in the bone marrow a larger number pass into venous trunks and in consequence through the right ventricle and pulmonary aorta to the lungs. This would seem to be the simple explanation of the fourfold increase in the combined number of pulmonary giant cells and giant nuclei in these experiments. Judging from my preparations megakaryocytes are slightly more numerous in the lungs of kittens as compared with adults.

Howell and Donahue state that in their microscopical specimens of the lungs of the normal cat there may be as many as forty megakaryocytes in an area of 100 sq.mm. If the term "megakaryocyte" was used, as appears to be the case, to designate naked giant nuclei, large partition products of such nuclei in various stages of degeneration, giant nuclei with small amounts of granular cytoplasm, as well as occasional cells with considerable amounts of cytoplasm, then the appearance of such "cells" in my preparations fully confirms this observation. In occasional low power fields (mag. $\times 90$) from four to five "megakaryocytes" can be seen. However, in similar fields of femoral bone marrow from the same cat, from ten to thirty approximately normal megakaryocytes of various sizes can be readily counted. On the basis of such comparative data alone it would seem that the lungs could have only very minor functional significance with reference to platelet production. Admitting an actual slight net increase in the number of plate-

lets in the blood leaving the lungs as compared with that entering the lungs, the accession seems most reasonably explainable as the result of liberation of granular fragments of cytoplasm persistent on giant nuclei swept into the lungs from the pulmonary artery, fragmentation of the cytoplasm of occasional approximately normal migrant megakaryocytes degenerating in the capillaries of the lungs, and fragmentation of numerous intracapillary giant platelets into platelets of average size. Such a possible slight functional addition to the number of platelets in the blood leaving the lungs is largely canceled by the disintegration of a certain number of platelets in the alveolar capillaries.

In the bone marrow the megakaryocytes originate from myeloblasts in the intervascular stroma; then pseudopods project into the venous sinuses; occasional cells and the nuclei of spent megakaryocytes may subsequently also gain entrance to the venous sinuses. There is no evidence in sections of the lungs of similar megakaryocyte formation. Nor is there evidence of intracapillary development of immature megakaryocytes. The presence of lymphocytes of various sizes, the cytoplasm of which with this technic may show variable amounts of azurophilic granules, may give the misleading impression of transitional stages between myeloblasts and giant cells.

Furthermore, since the same varieties of giant nuclei with adherent remnants of granular cytoplasm as appear in the alveolar capillaries occur also in the pulmonary aorta and its successively smaller intrapulmonary branches, there would seem to remain no plausible support for the suggestion that the naked and fragmenting nuclei of the terminal capillary mesh represent degenerative stages of megakaryocytes which originated from local myeloblasts.

The most important finding in the present investigation concerns the presence in the blood of the right ventricle, the pulmonary artery and the inferior vena cava of the normal kitten and full-grown cat, both in sections and smears, of a small number of megakaryocytes in various regressive stages, including apparently normal giant cells with large amounts of

granular cytoplasm and segmenting pseudopods, giant nuclei with small amounts of granular cytoplasm, naked giant nuclei and smaller partition products of such, and isolated pseudopods or giant platelets.

The lungs do not appear to be a locus of megakaryocyte production; they are merely a filter for megakaryocytes and their degenerative stages, in the form mostly of giant nuclei, which may have escaped into the venous sinuses of the bone marrow. The contribution of platelets in the lungs would seem to be of only very minor functional importance and only incidental to the platelet producing activity of the megakaryocytes of the bone marrow. As regards the megakaryocytes and their nuclei, which may pass through the heart to the pulmonary artery, the more important function of the lungs would seem to relate to the detention and destruction of these potential emboli before they can enter the left side of the heart where they could then be carried to the cerebral capillaries.

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PLATE 1

EXPLANATION OF FIGURES

1 Distorted megakaryocyte nucleus in alveolar capillary. Attached to one end is a cap of cytoplasm with characteristic reddish purple granules. Lung of adult cat. Dominici fixation.¹ Wright's stain.² Mag. $\times 1200$.

2 Bifurcated nucleus in dividing capillary. To the end of the lower nuclear limb is attached a fragment of granular cytoplasm. Cat.

3 Alveolar capillary with three fragments of a degenerating megakaryocyte nucleus and a large granular plastid (platelet). The smallest of the three nuclear bodies is in a later stage of dissolution. The plastid presumably represents the originally attached cytoplasmic remnant of an intact megakaryocyte. Cat.

4 Alveolar capillaries with three huge blood platelets and one red blood corpuscle. Lung of 2-month-old kitten.

5 Alveolar capillary with polymorphous megakaryocyte nucleus and three adjacent blood platelets with reddish purple granules. The polar cap of cytoplasm contains identical granules. Kitten.

6 Alveolar capillary with apparently normal small megakaryocyte and an adjacent platelet. This nucleus is invested at one end with a considerable amount of granular cytoplasm. Kitten.

7 Approximately normal small megakaryocyte in alveolar capillary. The cap of cytoplasm at the lower end contains relatively few granules. Kitten.

8 "Basket" nucleus, with small remnants of granular cytoplasm, in pre-capillary arteriole. Kitten.

9 Polymorphous megakaryocyte nucleus, with meager remnants of granular cytoplasm, in arteriole. Cat.

10 Degenerate giant-cell nucleus, with polar remnants of granular cytoplasm, in the lumen of a large branch of the pulmonary artery. The wall of this artery has a width equal to the greatest length of this megakaryocyte. Adjacent to the nucleus, on either side, are a red blood corpuscle and a lymphocyte. Cat.

11 and 12 Two adjacent sections of the same megakaryocyte in the lumen of a large bronchial branch of the pulmonary artery. Cat.

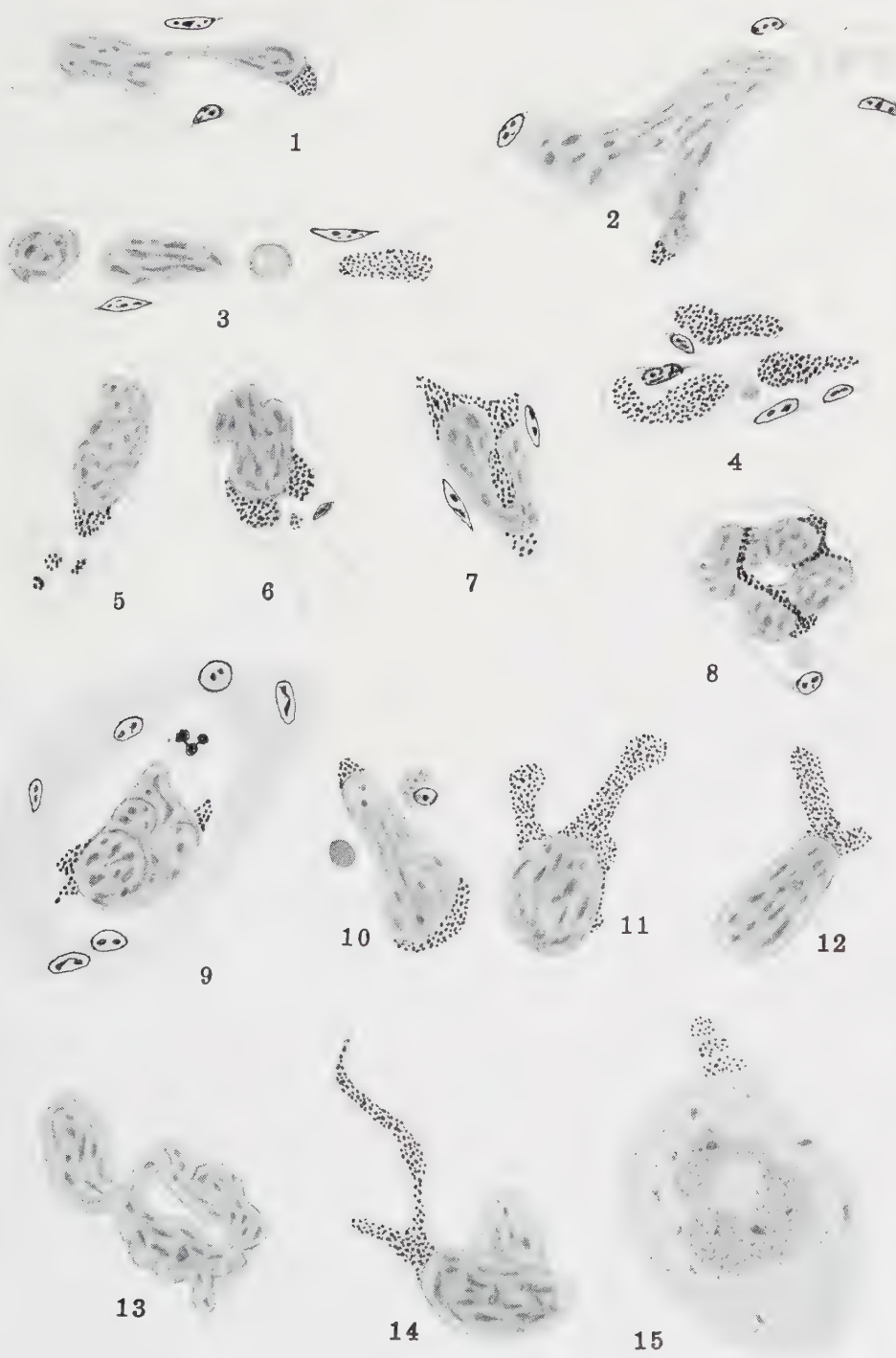
13 Typical basket nucleus from the same artery. The two meager remnants of cytoplasm lack granules.

14 Megakaryocyte in the lumen of the same large bronchial branch of the pulmonary artery. Attached to one pole of the bilobed nucleus is a projecting mass of granular cytoplasm to which is attached an enormously long slender granular pseudopod.

15 Typical megakaryocyte from smear preparation of blood from pulmonary aorta of adult cat. Wright's stain. Mag. $\times 1200$.

¹ Sublimate-tincture of iodine-formol mixture. Saturated aqueous solution (37%) of mercuric chloride, 20 parts; add tincture of iodine, 2 parts; filter the mixture; add 2 parts of formol to filtrate; transfer tissues after fixation direct to 70% alcohol.

² The microscopical preparations were made by the laboratory technician, Miss Ethel Venning.



THE FETAL MEMBRANES OF THE KANGAROO RAT, DIPODOMYS, WITH A CONSIDERATION OF THE PHYLOGENY OF THE GEOMYOIDEA

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ONE TEXT FIGURE AND THREE PLATES (FOURTEEN FIGURES)

The family Heteromyidae includes five living genera: *Dipodomys* (kangaroo rat), *Microdipodops* (gnome mouse), *Perognathus* (pocket mouse), *Liomys* and *Heteromys* (spiny pocket mice). The genus *Dipodomys* is sometimes separated into two different genera on the basis of the number of toes in the foot; the four-toed forms being then classified as *Dipodomys* and the five-toed forms as *Perodipus*. All material on the kangaroo rat used in this study is classified as *Dipodomys* according to the one nomenclature for the group.

The heteromyids show several specializations, among them being fur-lined cheek pouches as a special carrying adaptation present in but one other rodent family, the Geomyidae. *Dipodomys* has highly inflated mastoids and auditory bullae giving the head an appearance quite out of proportion to the body size. This development also occurs in *Microdipodops*. Wood ('35) notes that a correlation exists between this specialization and the development of long hind legs with which these two forms carry out ricochet locomotion. They possess fused cervical vertebrae which he suggests may be a later development also arising from this type of locomotion. Because of these structures the family is an interesting one and since no description has been made of the early development, especially with respect to the formation of the fetal membranes and the placenta, it was thought advisable to make

* Aided in part by a grant from the Wisconsin Alumni Research Foundation.

a study of the development of the fetal membranes of one of these forms. *Dipodomys* was chosen because of the relative ease with which material could be obtained. There are distinct differences between the genera of the family, however, and these are treated in the discussion.

Considerable work has been done on the Rodentia relative to various placental problems, and all of this is so well summarized by Mossman ('37) that an extensive review of the literature here is unnecessary. In this monograph Mossman shows that, on the basis of fetal membranes, the heteromyids occupy an intermediate position on the phylogenetic tree for rodents. Beyond a necessarily brief treatment of the type of implantation and general appearance of the placenta no more on these forms is given. The only other work on the Heteromyidae was an abstract by Lee ('18). In this he briefly describes the implantation and amnion formation in *Dipodomys*. It has been possible to study these slides and many others of Doctor Lee's through the courtesy of the Department of Anatomy of the University of Minnesota.

MATERIALS AND METHODS

Material, other than that in the Lee collection, was collected in the field and the entire reproductive tract excised and fixed immediately in Bouin's or alcohol-formol-acetic fixatives. Some was dehydrated by the alcohol method, but most was run up by the dioxan technic. The stains used were iron trioxynaematein counterstained with Triosin, and Delafield's haematoxylin (Harris' modification) counterstained with erythrosin and orange G. Serial sections were made of the early stages and interrupted serials of the later. Nearly all were cut at 8 μ . The material included a series of stages from the fertilized egg to nearly term. The presence of two recently fertilized eggs (two pronuclei in each egg) in the ampulla of the oviduct of one specimen having a recent post-partum uterus indicates that *Dipodomys* may have two or more litters a year. The breeding season is known to extend from January into August.

Part of this work was done in the Department of Zoology of the University of Utah where I am indebted to Dr. W. W. Newby for much aid in the early stages of the study. Particular appreciation is due Dr. H. W. Mossman of the University of Wisconsin for the use of figures 2, 3, 6 and 11 which appeared in his monograph previously cited ('37), for aid in the other photography, and especially for considerable critical advice during most of this work.

DESCRIPTION

1. The uterus

The uterus of *Dipodomys* is of the duplex type in which the two horns communicate with lateral vaginal recesses by separate openings. No uterine glands are present. The myometrium is composed of two distinct muscle layers, an outer longitudinal and a slightly thicker inner circular layer. Between the two is a prominent vascular area which transmits many of the larger vessels within the uterus. Of the other heteromyid forms studied, *Microdipodops* and *Perognathus*, both have a duplex uterus but only the latter species has uterine glands. This plays an important part in the formation of the placenta of *Perognathus* as will be noted later. The uterus of *Geomys* (family, *Geomyidae*) is much like that of *Dipodomys*.

2. Implantation and the yolk-sac placenta

Implantation in *Dipodomys* is on the antimesometrial side, with the embryonic disc oriented toward the mesometrial side. The stage showing the first contact of the embryo with the uterine epithelium is lacking, but in all probability it would appear much as in *Geomys* where first contact is made by a morula stage (Mossman and Hisaw, '40). In *Dipodomys* the endometrium undergoes hyperplasia on the antimesometrial side. It is in this tissue that the blastocyst embeds itself and comes to lie almost completely surrounded by maternal tissue. Only a small portion of the blastocyst re-

mains exposed to the uterine lumen. This constitutes, therefore, incomplete interstitial implantation. In figure 2, the small area exposed to the uterine lumen can be seen. A small amount of debris lies on the amnio-embryonic area,¹ and surrounding the trophoblast more may be seen, probably arising in part at least from the action of the trophoblast. As can be seen, the uterine epithelium is beginning to show signs of degeneration, some places becoming loosely stratified, while in others it is already sloughing into the lumen. The clear part immediately beneath the epithelium represents an edematous area. This is much more apparent just adjacent to the embryo than on the mesometrial side. Not showing in the figure is a limited area of edematous tissue adjacent to the circular muscle layer on the mesometrial side. This area extends about one-third the distance to the uterine lumen at this time.

The process of inversion of the germ layers (entypy), diagrammatically represented in figure 1, begins before the embryonic disc can be made out as a definite structure. In figures 1a and 2, the amnio-embryonic area is just beginning to be differentiated and is seen to be sinking into the yolk-sac. Vestigial folds appear (fig. 1b) and constrict the opening to the uterine lumen. This constriction forms the amniotic tube connecting the potential amniotic cavity with the uterine lumen (fig. 3). Deepening of these folds succeeds in closing the amniotic tube at the end nearest the embryo, establishing, thereby, the amniotic cavity next the embryo and another cavity, the epamniotic, on the uterine lumen side (fig. 1c). Mesenchyme has been proliferated from the undifferentiated tissue of the embryonic disc concurrent with the processes just described. This tissue lies as a network between the endoderm of the yolk-sac and the ectoderm of the amniotic tube. About the time that the amniotic and epamniotic cavities

¹ The disc-like structure seen in figure 2 is probably not just tissue of the embryonic disc, but undoubtedly includes tissue which will contribute to the formation of the amnion and epamnion. Hence this has been arbitrarily designated amnio-embryonic area, and the term, embryonic disc, has been reserved for that tissue which gives rise to the embryo proper.

are formed, spaces appear in the mesenchyme to form the exocoelom (figs. 1c and 1d). Enlargement of this coelom gradually separates the amniotic and epamniotic cavities. Proliferation of trophoblast cells gradually plugs the opening from the epamniotic cavity to the uterine lumen (figs. 1c, 1d

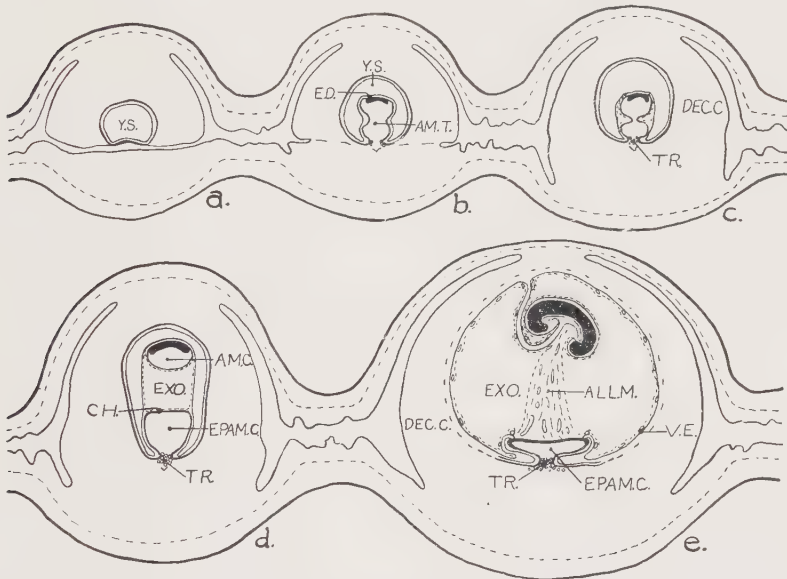


Fig.1 Diagram to show the processes of implantation and inversion of the membranes until complete inversion is accomplished, i.e., disappearance of the bilaminar omphalopleure as a definite membrane at the limb-bud stage (e), and migration of its component trophoblast cells into the surrounding maternal tissue. The figure also shows the method of formation of the decidua capsularis. The different stages are not all drawn to the same scale, the younger stages being more enlarged to show the structures desired. Mesometrial side down. ALL.M., vascular allantoic mesoderm; AM.C., amniotic cavity; AM.T., amniotic tube; CH., chorion; DEC.C., decidua capsularis; E.D., embryonic disc; EXO., exocoelom; EPAM.C., epamniotic cavity; TR., trager; V.E., vascular endoderm of the yolk-sac, Y.S.

and 1e). These cells form a small knot, the trager, which remains clearly differentiable until relatively late in pregnancy, acting as the vanguard of trophoblast tissue as it erodes the maternal decidua (figs. 4 and 6). By the time the amniotic and epamniotic cavities have been formed, the embryo is in the primitive streak stage.

The epamniotic cavity never becomes very large; in figure 5 it is seen at about its greatest size. The amniotic cavity, however, enlarges rapidly at first, providing an area in which the embryo can subsequently develop. This enlargement is made possible through growth into the surrounding highly edematous maternal tissue. The edema is especially marked in the area just antimesometrial to the embryo of the stage shown in figure 3. The tissue here soon becomes a loose connective tissue network in which the remaining capillaries appear to be suspended. These capillaries are made more prominent through a slight swelling of their endothelium. The picture is first one of a corona of vessels leading away from the vicinity of the embryo toward the larger vessels in the myometrium. Growth of the embryo soon compresses much of this tissue so that it finally appears much more firm.

In figure 3, the tissue just beneath the remnant of the uterine lumen shows the slight edema present at this stage on the mesometrial side. Soon this edematous process becomes very extensive (fig. 4). It is into this area that the *träger* advances in the development of the fetal portion of the allantoic placenta. The blood vessels in this area develop swollen endothelium early in the process and form a conspicuous nest of capillaries which is the first group of vessels to be eroded by the *träger*. The result of such eroding action is shown in figure 6, where surrounding the pale, tongue-like projection of *träger* tissue is a darkly staining mass of extravasated blood.

Reference again to figure 1 will show the fundamentals in the formation of the decidua capsularis. In "a" the embryo is implanted in the greatly hypertrophied antimesometrial tissue. Some growth of the tissue on the mesometrial side is also shown. As hypertrophy of the uterine connective tissue continues, the antimesometrial and mesometrial tissues approach each other, and with loss of the maternal epithelium, they fuse (b and c) and obliterate the uterine lumen. The embryo then lies completely enclosed by maternal tissue and the bilaminar omphalopleure lies pressed against the cap-

sularis. Large cells proliferate from the bilaminar omphalopleure into the capsularis. These apparently have a trophic function and because of their lighter staining cytoplasm and large size they can be distinguished in the capsularis until it disappears shortly before term. While these individual trophoblast cells can always be distinguished in the capsularis, the original trophoblastic layer remains as such only until the limb-bud stage (fig. 1e) at which time the bilaminar omphalopleure has completely disappeared as a structural entity. This complete disappearance is the final stage in the inversion of the membranes and at this time, then, the membranes of *Dipodomys* may be said to be completely inverted. The endoderm of the bilaminar omphalopleure does not continue as a definite membrane quite as long as does the trophoblast. The homogeneous, hyalin membrane (Reichert's membrane) between the trophoblast cells and endoderm cells of the bilaminar omphalopleure appears to be the product of the endoderm cells. It remains as the internal boundary of the capsularis after the other constituents of the bilaminar omphalopleure have disappeared.

Vascular allantoic mesoderm makes contact with the epamnion at an early limb-bud stage to establish the chorio-allantoic placental circulation. Until this time, then, all the nutrition of the embryo has been carried on by the yolk-sac placenta. The tissue actively concerned in the functions of this placenta is the inverted vascularized portion of the yolk-sac. The tissues which probably play just a passive role are those making up the bilaminar omphalopleure, i.e., the trophoblast and endoderm (both of which essentially disappear early) and Reichert's membrane (which remains as long as there is a capsularis). After the capsularis disappears (nearly term) the vascular endoderm of the yolk sac comes into direct contact with the uterine mucosa.

The vascular inverted yolk-sac undergoes but little change in structure throughout gestation. Beginning as a simple smooth membrane with columnar or cuboidal endodermal cells portions of it soon become specialized by development of villi.

These villi are folds of endoderm containing a vascular core (fig. 9). They are found in a limited area around the placental disc (chorio-allantoic placenta, fig. 13), and by their structure greatly increase the absorptive area of the yolk-sac placenta. It is in this limited region that much debris, derived from the decidual tissue of the capsularis and basalis, is found. It is also a natural well into which nutritive substances getting into the yolk-sac may possibly be found in greater amounts.

3. The chorio-allantoic placenta

When the epamnionic cavity is formed, it becomes possible to distinguish two portions of trophoblast having different functions. One is the portion which forms the träger and walls of the epamnion, and the other is the trophoblast of the bilaminar omphalopleure. Of the former, only a small part is lined with mesoderm of the exocoelom (figs. 1d and 4). This area is homologous to the true chorion of other mammals, and it is this portion of the epamnion that becomes vascularized by the allantoic vessels (figs. 1e, 6 and 7).

Vascular allantoic mesoderm reaches and makes contact with the epamnion by the limb-bud stage. Apparently stimulated by this contact, and the blood supply it brings, the cells of the epamnion begin to proliferate rapidly. The cavity of the epamnion becomes obliterated, and a solid mass of trophoblast tissue begins to develop on the surface of the hypertrophied uterine tissue (fig. 6). Preceding the main mass of the träger is a tongue-like projection of this lightly staining tissue apparently forcing its way into the darker staining maternal tissue. Immediately adjacent to the träger (in the same figure) the maternal tissue is edematous and into this the fetal tissue proliferates. The more darkly staining portion at the base of the main mass of träger tissue is maternal blood derived from the capillary net noted in the previous discussion. As this capillary net was denuded of its endothelium, maternal blood was freed into the tissue and began flowing in sinuses lined by the trophoblast. Such sinuses may

be seen as dark spots in the lightly staining trophoblast tissue of the figure.

Also evident in figure 6, and under higher magnification in figure 7, is a difference in staining reaction of parts of the trophoblast tissue derived from the epamnion. That part in contact with the vascular allantoic mesoderm, which was the true chorion, is present as a thin line of slightly darker staining cells. This tissue contains many mitotic figures and appears generally more virile than the other trophoblast. It is this portion which forms the main vascular labyrinth of this placenta. It probably contributes many cells to the trophoblast tissue which makes up the remainder of the fetal portion of the placenta.

In the development of this placenta the main changes are brought on by proliferation of fetal tissue. This continues until a cup-shaped disc is formed, the base resting on maternal tissue, the convex sides being in close proximity to the yolk-sac villi and the concave embryonic surface being vascularized by the allantoic mesoderm (fig. 13). As the placenta approaches maturity the ridge of the cup on the embryonic surface becomes flattened inward by the pressure of the fetus. The fetal tissue has increased greatly in volume while the maternal tissue has been reduced by only about one-half. This reduction of maternal tissue is relatively less than is seen in most rodents.

In its finer morphology the fetal placenta is divisible into three zones (figs. 12 and 13). One is the labyrinth on the fetal surface, which is the only portion traversed by fetal blood vessels. It makes up about one-third of the fetal tissue. The fetal blood vessels dip down into the labyrinth at various places surrounded by a small amount of allantoic mesoderm forming the fetal villi (fig. 10). Their path downward to the base of the labyrinth is relatively straight, and near the base they turn and lead up toward the fetal surface again but in a very tortuous manner. This path interdigitates with irregular trophoblastic sinusoids filled with maternal blood. In many places in the maternal placenta, however, the fetal endothelium appears to be bathed directly with the maternal blood,

no trophoblast layer lying between them, or else it is so thin that it cannot be demonstrated.

On the fetal surface large sinuses in the trophoblast are filled with maternal blood. These sinuses are connected to one or two large vessels, thought to be arteries, which may be traced from near the myometrium through the placenta to the surface. From the sinuses, maternal blood percolates down through the trophoblast tubules and where the trophoblast appears to be absent the blood flows directly over the tortuous fetal capillaries.

The second zone of fetal material is a zone of large, mostly mononuclear trophoblast cells. The cytoplasm stains deeply in the fetal portion of this zone. The nuclei throughout are large and vesicular (fig. 12). This zone is traversed by numerous channels, filled with maternal blood, leading from the labyrinth. They appear to be venous channels which unite with similar channels in the basal zone and lead away into vessels in the myometrium. They have an endothelium-like lining, although from where this comes it is impossible to say. In the earlier stages, the maternal blood was found in trophoblastic tubules, but on further development some of these tubules in the trophoblastic tissue are lined by squamous cells. This lining may have arisen from stretched out trophoblast cells or possibly even from migratory cells from the maternal blood vessels themselves.

The basal zone makes up about one-third of the fetal tissue. It is characterized by smaller cells with more chromatic nuclei than those previously described, and endothelial-lined maternal vessels are more numerous here. At the border of the placental disc these vessels are especially numerous forming a dense network leading down to vessels in the myometrium.

The decidual tissue, other than the capsularis, forms the base of the placental disc (fig. 13). This decidual tissue begins as a growth of maternal connective tissue at the time of implantation. It reaches its maximum growth at about the time shown in figure 6. From this time on there is a gradual

reduction through action of the trophoblast, but at term there still remains relatively about half of the amount of the original tissue. The decidual tissue at term is mostly necrotic and forms a rather definite zone of separation along which cleavage of the placenta occurs at parturition.

DISCUSSION

1. Comparison of the three genera of the Heteromyidae

Dipodomys and Microdipodops have almost identical types of implantation of the blastocyst in that it comes to lie practically completely interstitial in the antimesometrial tissue (fig. 2). Only a small portion of the blastocyst, which has been called the amnio-embryonic area, is exposed to the uterine lumen. The embryonic disc is oriented toward the mesometrial side in both genera. Inversion of the germ layers begins in *Dipodomys* before the blastocyst is completely embedded and before the embryonic disc is a definite structure. This inversion is completed by the limb-bud stage when the bilaminar omphalopleure has disappeared and the vascular yolk-sac comes in contact with the decidua capsularis. *Microdipodops* has already undergone partial inversion of the membranes in the earliest embryo studied. Here the embryo is in an early primitive streak stage in which the amniotic cavity and the exocoelom are already formed, and the epamniotic cavity is just being closed by a small growth of Träger cells. This embryo is practically identical with a comparable stage of *Dipodomys* and it seems very likely that inversion would begin about the same time in both forms and that amniogenesis would probably be accomplished by means of vestigial folds in *Microdipodops* just as it is in *Dipodomys*. In a later stage of *Microdipodops* the membranes are completely inverted and the relationship is practically identical with a comparable stage in *Dipodomys*. In both of these genera a decidua capsularis is formed.

The earliest available stages of *Perognathus* were poorly preserved so it is impossible to say just when inversion

occurs in this genus. Implantation is on the antimesometrial side and practically completely interstitial as in the other heteromyids. Inversion of the membranes in an early primitive streak stage is comparable to the same development in *Dipodomys* and *Microdipodops*. However, by this stage the decidua capsularis in *Perognathus* is very thin compared to the other genera, being only about five or six cells thick. Attachment of the capsularis to the basalis is by a very thin area. In a neural groove stage (fig. 8) the capsularis has disappeared and the bilaminar omphalopleure is represented by a few scattered cells along a thin hyalin, acellular membrane which is thought to be Reichert's membrane. To all practical purposes then, this represents complete inversion of the germ layers at a neural groove stage, whereas complete inversion in *Dipodomys* was not accomplished until a limb-bud stage. In figure 8, a narrow decidua parietalis can be seen with but a few necrotic cells on its edges indicating that the decidua capsularis, which attached there, did not persist long and left but a few necrotic cells in the uterine lumen. In this neural groove stage small cells are seen penetrating the uterine epithelium in all areas. These are thought to be trophoblast cells which give rise to the prominent giant cells seen just under the epithelium (fig. 11) in the more mature stages of *Perognathus*. Large cells in this position are found in the heteromyids only in this genus.

In the development of the inverted yolk-sac *Microdipodops* and *Dipodomys* are practically identical, having rather simple villi formed from a fold of endoderm containing a capillary loop of the vitelline vessels (fig. 9). These villi are found in both forms in a limited region around the allantoic placental disc (fig. 13). In all other areas the yolk-sac is a more or less avillous structure in contact with the decidua capsularis until this disappears and then it becomes smoothly pressed against the uterine mucosa. In *Perognathus* similar villi are found in the region of the placental disc but are not so numerous. They are striking in appearance in that a considerable deposit of hemosiderin is found in the

endoderm cells. Over the remainder of the inverted yolk-sac in *Perognathus* a very intimate apposition of yolk-sac and maternal tissue is found (fig. 11). This is brought about through interdigitations of fetal and maternal tissue in which the yolk-sac lies over nodular projections of the uterine mucosa and forms a sort of hollow villus between each of these projections. The structure of these simple villous projections can be best appreciated by comparing those in figure 11 with the solid villi shown in figures 9 and 13. This type of villous formation in *Perognathus* is possibly to be correlated with the very early disappearance of the capsularis, with the relatively early complete inversion of the germ layers, and with the presence of uterine glands found in this genus and in neither of the other two heteromyids studied.

No allantoic vesicle is present in any of the three forms studied.

The allantoic placenta of *Dipodomys* was the only available full-term placenta of the three genera studied. However, comparing similar ages of *Microdipodops* and *Dipodomys* shows these to be very much alike in finer as well as gross structure. The oldest stage available for *Microdipodops* was one in which the gestation sac in the fixed condition measured 5×9 mm. Small projections from which the toes would develop are just visible on the foot pads of the fore-limbs of the fetus; the eyelid folds have not yet begun to grow over the eye; no hair follicles are present anywhere; the fetal blood appears to be all nucleated. By this stage in *Microdipodops* the area in the fetal placenta into which the fetal blood vessels have penetrated includes less than one-third of the fetal tissue of the placenta (fig. 15), as it does in a comparable age in *Dipodomys*. If the development proceeds as in *Dipodomys* it means that this area, which will become the labyrinth of the mature placenta, will make up about one-third of the fetal placenta (figs. 12 and 13). Whether the trophoblastic tissue beneath this pro-labyrinthine area in *Microdipodops* will differentiate into two distinct zones as it does in *Dipodomys* is unknown (perhaps it will remain

undifferentiated as in *Geomys*). But, again in *Perognathus* a deviation from the others is seen. Here, the allantoic mesodermal villi project down to the base of the fetal placenta as can be seen in figure 14. The gestation sac of this stage measured 7×12 mm. in the fixed state. In the fetus the eyelid folds are just beginning to be definite; shallow hair follicles are present for the vibrissae; the toe projections from the foot pad are slightly more prominent than in the oldest stage in *Microdipodops*. Altogether it is an older specimen than the one of *Microdipodops*. The labyrinth in this stage of *Perognathus* is not limited to a part of the placenta, but instead, the fetal blood vessels traverse all the fetal placenta. The labyrinth is not so complexly trabeculated as in a comparable stage of *Dipodomys* and scattered throughout the placenta are clumps of pale staining trophoblast not seen in the labyrinth of other heteromyids.

No accessory placenta is present in any of the forms studied.

The giant cells of *Dipodomys* and *Microdipodops* are all thought to be trophoblastic in origin and are seen in the decidua capsularis of both forms where this structure becomes composed almost entirely of giant cells as the trophoblast erodes the maternal tissue. Those in *Microdipodops* are larger and cause the capsularis to appear much as this structure does in *Geomys* shortly before it completely disappears. The mature fetal placenta of *Dipodomys* (not available for *Microdipodops*) has many giant cells making up the basal zones, especially the middle zone (fig. 12). Giant cells of *Perognathus* have been noted as lying under the uterine epithelium (fig. 11) and are thought to originate in this area solely from the trophoblast. These are the largest of all giant cells in the three genera, some measuring up to 40×50 μ . Other giant cells are present in the maternal decidua on the mesometrial side. These cells appear to be maternal in origin and can be seen in all stages of growth in the connective tissue adjacent to the muscularis. Small and inconspicuous cells found mostly around the blood vessels in the myometrium in *Dipodomys* appear to be comparable to the

nephrophagocytes of Ancel and Bouin and appear to be of maternal origin.

2. Phylogenetic relationships on the basis of the fetal membrane data

From the preceding discussion it is evident that Microdipodops and Dipodomys are very similar in all the known stages of membrane formation, not only to each other, but as noted below, to Geomys as well. This is to be expected since the geomyids and heteromyids are generally thought to have a common, but as yet unknown ancestor. Geomys has a slightly more superficial method of implantation (Mossman and Hisaw, '40) than the heteromyids, but as gestation progresses it comes to be more and more like Dipodomys and Microdipodops. It has a decidua capsularis which is largely replaced by trophoblast giant cells until it looks much like the same structure in Microdipodops. The full-term allantoic placenta is much like that in Dipodomys except that the labyrinth makes up about half of the fetal tissue. The remainder of the fetal placenta in Geomys is not made of two zones as in Dipodomys but of a single zone of trophoblast cells in which acellular spaces are present, apparently the result of the disappearance of some small areas of trophoblast cells. These cell-free areas in the trophoblast are not seen in Dipodomys nor are there ever any clumps of trophoblast present which resemble those areas seen in the immature geomyid placenta. However, such areas of differentially staining trophoblast can be seen in the placenta of Perognathus (fig. 14). In a slightly later stage these areas of light staining cells have disappeared and leave large, cell-free areas much like those in the geomyid placenta. In Perognathus a few of these spaces are bridged by cords of trophoblast cells carrying some fetal allantoic vessels to the base of the fetal placenta, but in Geomys the labyrinth is more limited by this stage.

A comparative study of the inverted yolk-sac reveals interesting differences in the structure of the villi. In Perognathus most of these are of the simple, hollow type described previ-

ously as interdigitating with the uterine mucosa (fig. 11). In a limited area near the placental disc are found a few villi of the solid type formed of a fold of endoderm around a core of mesoderm containing small vitelline vessels. In *Geomys*, folds of the inverted yolk-sac project irregularly into the capsularis in a much simpler and less regular manner than the hollow villi of *Perognathus* interdigitate with the uterine mucosa. *Geomys* also has many complexly branched, solid villi near the placental disc. Similar solid villi may be found in *Dipodomys* (figs. 9 and 13) and *Microdipodops* near the placental disc, but in these genera no infolding into the capsularis is present. Whether such varying relations of the yolk-sac to maternal tissue are of any significance in an evolutionary sense is impossible to say as yet.

Fetal membrane data from the superfamily Geomyoidea indicate that a close relationship exists among *Geomys*, *Microdipodops* and *Dipodomys*. *Geomys* implants more superficially than the other two forms, but by the time the allantoic placental circulation is established in the three forms (limb-bud stage) their development has become very similar and continues to be so from this time on. Small variations in the yolk-sac noted above and in the allantoic placenta exist but, so far as the problem is understood at present, these are not thought to be of any great significance. It is difficult to orient *Perognathus* in its true relationships to the other members of the superfamily partly because of the lack of suitable material on the early stages and partly because of its apparent divergence from the typical heteromyid development. Implantation appears to be almost as completely interstitial as in *Dipodomys* and *Microdipodops*. However, the decidua capsularis in *Perognathus* is very thin from the start and disappears very early, except for a remnant near the decidua parietalis and basalis. Concurrent with the disappearance of the capsularis is the disappearance of the bilaminar omphalopleure giving rise to a completely inverted yolk-sac at a very early stage (fig. 8). It is generally thought that interstitial implantation and an early disappearance of

the bilaminar omphalopleure are both highly specialized conditions in rodents. On the basis of these factors *Perognathus* might superficially seem to be more specialized. However, there are also characters present in this genus which resemble those of more primitive rodents. One of these is the presence of giant cells, thought to be of trophoblastic origin, scattered diffusely just under the uterine epithelium (fig. 11). These cells are much like those seen in the rabbit where they are also thought to come from the trophoblast. Then too, giant cells, probably of maternal origin, are present in the decidua basalis of *Perognathus* but in no other known member of the superfamily Geomyoidea. These can be compared to the giant cells in the deeper portions of the basalis of the rabbit and the squirrel (Mossman and Weisfeldt, '39) where they are found mainly as sheaths around blood vessels and are thought to be partly of trophoblastic and partly of maternal origin. The thickness of the trophoblastic layer separating the fetal capillaries from the maternal blood makes the placenta of *Perognathus* definitely hemochorial even in as mature a stage as illustrated in figure 14. Comparable ages of *Dipodomys* and *Geomys* are by this time hemoendothelial in type in many areas and the trophoblastic layer, where it does remain, is very thin. The condition in *Perognathus* may be compared with the rabbit where the hemoendothelial condition of some areas of the placenta is found only relatively late in development. These more primitive characteristics may be due to parallelism which is so commonly seen in rodents or they may be indicative of other relationships the present material does not show. Until more and better material of *Perognathus* becomes available it seems quite impossible to place this genus in its correct position with respect to the other forms.

The interpretation of *Geomys*, *Microdipodops* and *Dipodomys* as being probably the more closely related forms on the basis of their fetal membranes is somewhat at variance with their position as determined from interpretations based on paleontological and morphological evidences as set forth by Wood ('35). He considered only the *Heteromyidae* and

therefore a comparison of the two methods of classification so far as *Geomys* is concerned is impossible. According to Wood's data he places *Dipodomys* lowest on the scale, next comes *Perognathus* and then *Microdipodops*. The exact position of the latter is questionable according to him, but in any event it is placed above *Perognathus*. Whether it lies closer to *Perognathus* or *Heteromys* (fetal membranes not studied in the present work) is the point of which he feels in doubt. On the basis of the fetal membranes *Perognathus* certainly does not lie between *Dipodomys* and *Microdipodops*. From an incomplete series on *Microdipodops* it appears that this form may be intermediate between *Geomys* and *Dipodomys* rather than more specialized than *Dipodomys* as Wood's data indicates. It appears significant that *Dipodomys* and *Microdipodops*, which resemble each other superficially, should be so much alike in their relatively protected fetal membranes. That this is probably not just a modification to similar habits they have developed, such as their mode of locomotion (jumping), is seen in the close resemblance to these forms of *Geomys*, a rodent of mole-like habits.

Placental and fetal membrane characters do not seem variable enough between species to make any distinction. No significant differences were noted in the following species studied: *Dipodomys merriami* and *D. ordii*; *Perognathus parvus*, *P. pencillatus*, and *P. longimembris*; the more mature specimens of *Microdipodops* were of *M. megacephalus*, the earlier were *M. pallidus*. The need for collection of material of *Perognathus*, *Liomys* and *Heteromys* is quite apparent and would do much to complete the picture of development in the heteromyids.

SUMMARY

1. Implantation in *Dipodomys* is incompletely interstitial and on the antimesometrial side of the uterus. The amnion is formed by vestigial folds, at which time an epamniotic cavity is also formed. This latter cavity is closed by the tr ager. Inversion of the yolk-sac begins before the embryo is wholly implanted and is completed by a limb-bud stage. The present

data indicates that the processes of implantation, amniogenesis and inversion of the yolk-sac in the other heteromyids studied, *Microdipodops* and *Perognathus*, are similar to the development in *Dipodomys*, but that in *Perognathus* inversion of the yolk-sac is completed earlier than in the other forms.

2. The allantoic placenta of *Dipodomys* is deciduous, cup-shaped discoidal, labyrinthine, hemochorial and very probably advanced from this in many areas to the hemoendothelial type.

3. The vitelline placentation is of the completely inverted type in *Geomys*, *Microdipodops*, *Dipodomys* and *Perognathus* with a variation among these genera in the intimacy of union between the yolk-sac and the maternal tissue.

4. A probable evolutionary distribution in the superfamily Geomyoidea is presented in the following order from the more primitive to the more highly evolved: *Geomys*, *Microdipodops*, and *Dipodomys*. The position of *Perognathus* is, as yet, questionable.

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PLATE 1

EXPLANATION OF FIGURES

2 Early disc stage of an implanting blastocyst of *Dipodomys*. The amnio-embryonic area (AM-E.) is seen slightly exposed to the uterine lumen (L.). The slight concavity of this area shows that inversion of the germ layers is commencing. Edema is present in the antimesometrial tissue (top) surrounding the blastocyst and along the uterine epithelium (U.E.). Some necrosis of the uterine epithelium is apparent. BI.OM., bilaminar omphalopleure; VIT.C., vitelline cavity, ♀ Lee 731, slide 1-1, section 47, sagittal, $13\frac{1}{2}$ μ . $\times 175$.

3 Slightly older blastocyst of *Dipodomys* showing progress in the inversion of the germ layers. The amniotic tube (AM.T.) is barely visible connecting the amniotic cavity with the uterine lumen (L.). The blastocyst is all but completely interstitial. Some edema is seen beneath the uterine lumen on the mesometrial side (bottom). Vestigial folds are constricting the amniotic tube. ♀ Lee 701, slide 3-2, section 57, transverse, $13\frac{1}{2}$ μ . $\times 175$.

4 Primitive streak stage of *Dipodomys*. The tr ager shows prominently; just mesometrial to this (below) is the remnant of the uterine lumen (L.) and below this an area of dilated capillaries with swollen endothelium. Note the edematous tissue surrounding the embryo. AM.C., amniotic cavity; EPAM.C., epamniotic cavity; EXO., exocoelom; Y.S., yolk-sac. *D. merriami* ♀ 6, slide 3 row 3, section 10, transverse, 8 μ . $\times 15$.

5 Neural groove stage of *Dipodomys*. Mesometrial side below. The decidua capsularis (DEC.C.) is formed; the decidua basalis (DEC.B.) is greatly hypertrophied, with marked edema in the lightly staining tissue on the side next the embryo. Maternal blood shows up as dark spots in this lightly staining tissue. The cavity just above the basalis is an artifact; just above this is the epamniotic cavity (EPAM.C.). DEC.P., decidua parietalis; EXO., exocoelom; L., uterine lumen; Y.S., yolk-sac. *D. merriami* ♀ 7, slide A4, line 2, section 8, sagittal, 10 μ . $\times 14.5$.

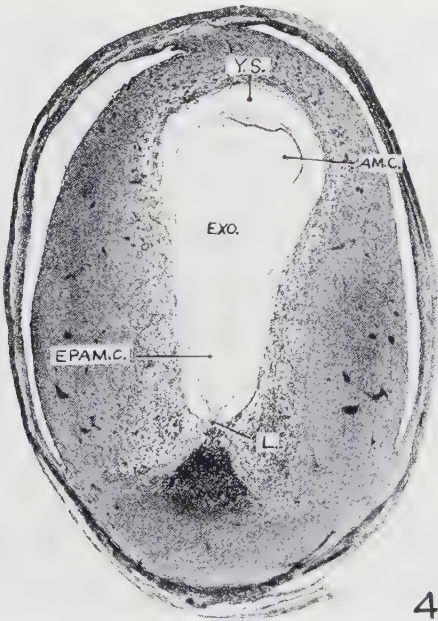
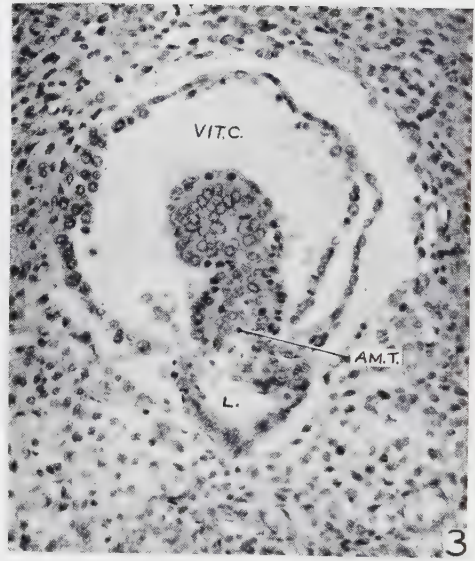
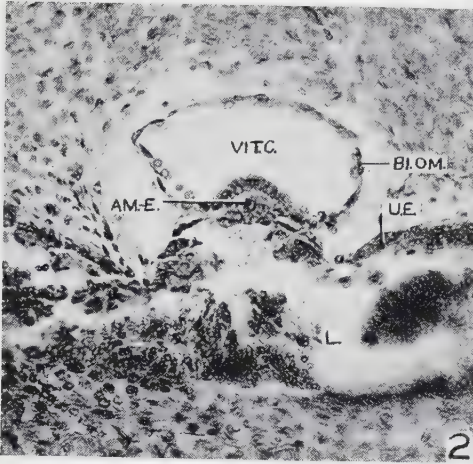


PLATE 2

EXPLANATION OF FIGURES

6 Embryo in gill-slit stage. The vascular allantoic mesoderm (ALL.M.) has contacted the chorion, the epamniotic cavity has been filled by proliferation of the lightly staining cells from the walls of the epamnion and the tr ger. Immediately around the tr ger (TR.) is the dark extravasated blood from maternal vessels the tr ger has opened; dark spaces in the light tr ger tissue are maternal blood sinuses (no endothelium). Mesometrial side at bottom. DEC.C., decidua capsularis; EXO., exocoelom; L., lumen. *D. merriami* ♀ 8, slide D 5, section 4, sagittal 7 μ . $\times 7$.

7 Magnification of a portion of an area of the allanto-chorion of figure 6. Allantoic mesoderm at top; CH., chorion; F.B.V., fetal blood vessel; TR., tr ger. $\times 210$.

8 Section of a neural fold stage of *Perognathus*. Note the almost complete loss of the bilaminar omphalopleure (BI.OM.), only a few scattered cells and what appears to be Reichert's membrane remain. Note the presence of uterine epithelium (U.E.) over all the lumen except just in proximity of the epamnion attachment (EPAM.C.). GL., uterine gland; Y.S., yolk-sac. *P. longimembris* ♀ 2, slide 6, section 6, transverse, 10 μ . $\times 13.5$.

9 Section showing the yolk-sac villi of *Dipodomys* near term, in the region of the placental disc (see fig. 13). From below upwards is uterine wall, uterine epithelium (U.E.), uterine lumen (L.), non-villous portion of the vascular endoderm (V.E.) of the yolk-sac (Y.S.), exocoelom (EXO), and villous portion of vascular endoderm of yolk-sac. Each villus contains a capillary core. Note the simple apposition of uterine mucosa and non-villous part of the yolk sac (contrast with fig. 11). *D. merriami* ♀ 9, slide H, sagittal, 7 μ . $\times 180$.

10 Enlargement of a portion of the placental labyrinth of the mature placenta of *Dipodomys* (see fig. 12) showing a single allantoic mesodermal villus carrying a fetal blood vessel (F.B.V.) from the surface to the base of the labyrinth. The area associated with this villus shows up as the more dense tissue in the central part of the figure. M.B.S., sinus containing maternal blood; G.C., giant cell zone. *D. merriami* ♀ 9, slide D, sagittal, 10 μ . $\times 95$.

11 Section of the yolk-sac villi of *Perognathus*. From the bottom upwards is endometrium, uterine epithelium (U.E.), uterine lumen (L.), vascular endoderm (V.E.) of yolk-sac, and exocoelom (EXO.). A large trophoblastic giant cell (TR.G.C.) is seen beneath the uterine epithelium. Note the complex interdigitations of yolk-sac and maternal tissue. These villi are found over most of the yolk-sac. *P. parvus* ♀ 665, slide E2, section 5 sagittal, 10 μ . $\times 85$.

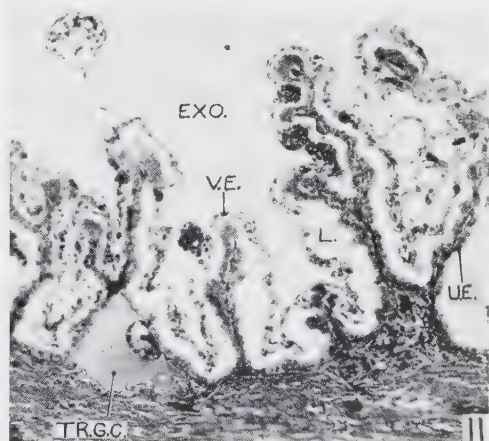
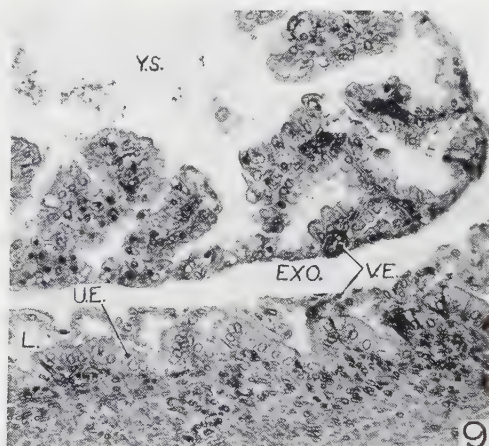
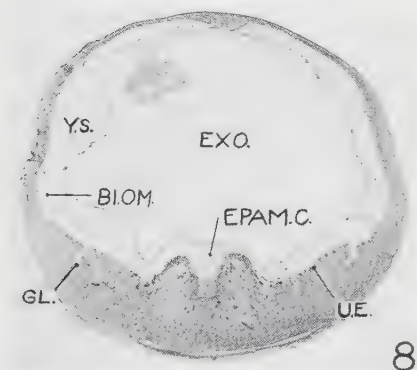
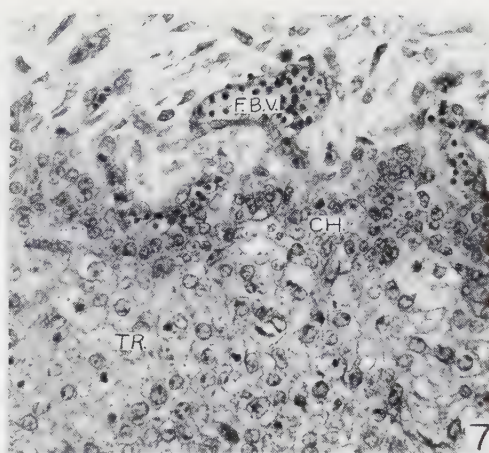
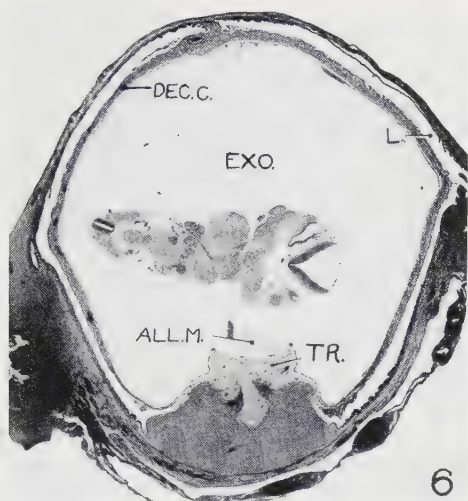


PLATE 3

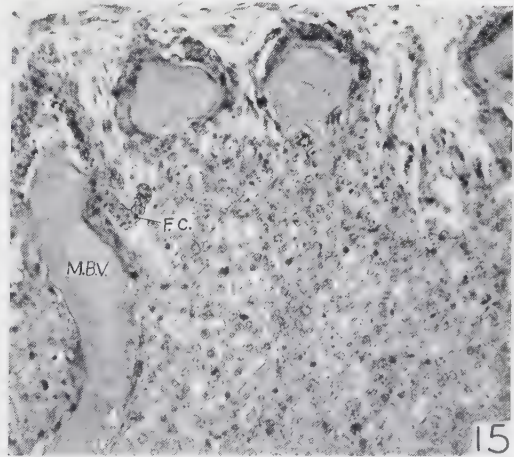
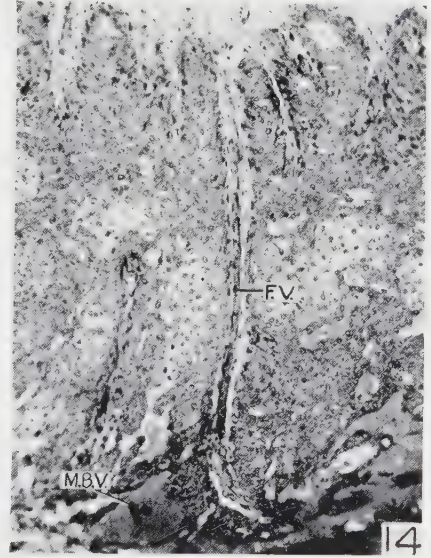
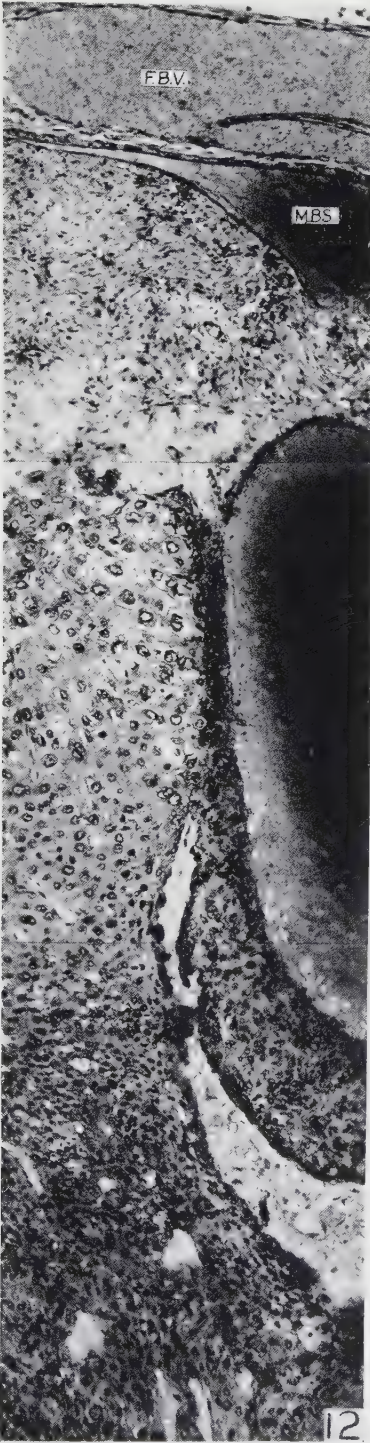
EXPLANATION OF FIGURES

12 High magnification of a portion of the placental disc of *Dipodomys* seen less magnified in figure 13. The large blood vessel on the right is thought to be an artery leading up to the sinus on the fetal surface. Note the three zones: top, labyrinthine zone consisting of vascular allantoic mesodermal trabeculae and some thin trophoblast in some areas; middle zone of large cells with no fetal but many maternal blood channels; lower zone of smaller cells and more numerous maternal blood channels. No maternal decidua appears in the figure. F.B.V., fetal blood vessel; M.B.S., sinus lined by trophoblast and containing maternal blood. Same slide as figure 10. $\times 90$.

13 Section of about half of the placental disc of *Dipodomys*. A large vessel, thought to be an artery filled with maternal blood, leads to the sinus (M.B.S.) on the fetal surface (top). F.B.V., fetal blood vessel; G.C.Z., zone of large trophoblast cells, the upper ones have more deeply staining cytoplasm; L.Z., labyrinthine zone; S.C.Z., zone of small trophoblast cells; VIL., yolk-sac villi; Y.S., yolk-sac; Z.S., zone of separation. $\times 13$.

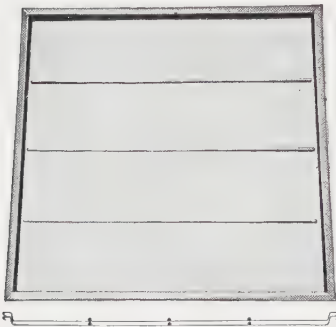
14 Section of the entire fetal portion of the placenta of *Perognathus* of stage described on page 116. Mesometrium below. Vascular allantoic mesoderm at the top carries fetal vessels (F.V.) to the base of the placenta. It is not limited to a single zone as in *Dipodomys*. Trophoblast-lined channels are fairly regular. Clumps of lightly staining trophoblast are prominent. M.B.V., maternal blood vessels lined by an endothelium-like layer connected to blood channels at the base of the placenta. P. (sp?) ♀ A, series D, slide 2, row 2, section 5, 8μ . $\times 105$.

15 Section of most of the fetal portion of the placenta of *Microdipodops* of the stage described on page 115. Mesometrium below. Top, vascular allantoic mesoderm which projects a limited distance into the placenta as in *Dipodomys*. Fetal capillaries (F.C., outline of labeled capillary slightly retouched) can be seen to be separated by trophoblast from the sinuses (M.B.V.) containing maternal blood. A large vessel, probably an artery, is seen on the left leading toward the sinuses. The remainder of the placenta, other than the labyrinthine zone, is composed of a single type of cell. *M. megacephalus* ♀ 1, series A, slide 4, section 2, 8μ . $\times 110$.



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THE CYTOLOGY OF NORMAL HUMAN SYNOVIAL FLUID ^{1, 2}

HOWARD C. COGGESHALL,³ CHARLES F. WARREN ⁴ AND WALTER BAUER
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FIVE FIGURES

Knowledge of the cytology of the normal human synovial fluid is meager (Hammar, 1894; Labor and Balogh, '19; Key, '28; Forkner, '30, and McEwen, '35). The earlier reports (Hammar, 1894 and Labor and Balogh, '19) indicate that the total cell count is low but give little information as to the types of cells and percentage of each. Although the more recent reports (Key, '28; Forkner, '30 and McEwen, '35) give both the total cell counts and percentage of various types of cells, in two instances (Key, '28 and Forkner, '30) no reference is made as to the number of fluids examined and in the other (McEwen, '35) only two normal fluids were so studied. From these incomplete data one is unable to speak with any certainty concerning the physiological variations encountered in normal individuals. Yet such knowledge concerning normal human synovial fluid cytology is necessary before one can undertake a comprehensive evaluation of the cytological characteristics of pathological synovial fluids. Such an evaluation must be based on the normal human fluid because of the marked variation in the total number of cells and the

¹ Publication no. 44 of the Robert W. Lovett Memorial for the study of crippling disease, Harvard Medical School, Boston, Massachusetts.

² The expenses of this investigation have been defrayed by grants from the John and Mary R. Markle Foundation and the Commonwealth Fund.

³ John White Browne Scholarship, Harvard Medical School, 1935-1937.

⁴ Dalton Scholar, Massachusetts General Hospital, 1933-1935.

percentage number of any one type of cell (Bauer, Bennett, Marble and Claffin, '30 and Warren, Bennett and Bauer, '35) observed in the various species of animals.

In order to establish the cytological characteristics of normal human synovial fluid, studies have been made on synovial fluid obtained immediately after death from patients without previous history of joint disease. A total of 185 fluid examinations was necessary because it soon became apparent that although some were normal, many were distinctly pathological. The variations from the normal were apparently due to infection or edema or a combination of the two. The influence of such factors on the cytology of the synovial fluid will be found in a subsequent report. Therefore, in establishing the cytological characteristics of normal synovial fluid, only fluids obtained from patients without a history of previous joint disease, dying from a non-infectious cause and without edema, were included.

METHODS

All samples of synovial fluid were obtained from knee joints. In order to minimize the possibility of contamination of the fluid with either tissue fluid or blood, a stylet-containing lumbar puncture needle was employed for all aspirations. The greatest and most constant quantity of synovial fluid was obtained by introducing the aspirating needle lateral to the patellar tendon and then directing it into position under the patella. Synovial fluid aspirations and cell counts were made within 30 minutes of the time of exitus. Total cell counts

Fig. 1 Illustrates the microscopic appearance of leukocytes when examined by the fixed stain method (Wright's) and the neutral red-Janus green supravital technique. Wright's stain being primarily a nuclear stain does not enable one to distinguish cytoplasmic granules except in the case of polymorphonuclear leukocytes and occasional cells with phagocytized particles. The supravital technique allows for a finer differentiation of the mononuclear phagocytes because the characteristic movements and staining of the cytoplasm can be observed.

The majority of the normal synovial fluid leukocytes are mononuclear phagocytes, therefore accurate differential cell counts necessitate the employment of the supravital technique.

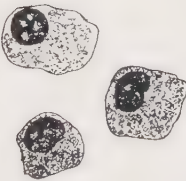
THE CELLS OF NORMAL SYNOVIAL FLUID		
	WRIGHT'S	SUPRA VITAL
POLYMORPHO - NUCLEAR		
MONOCYTES		
GLASSMATICYTES AND UNCLASSIFIED MONONUCLEAR PHAGOCYTE		
LYMPHOCYTES		
SYNOVIAL CELLS		
ERYTHROCYTES		

Figure 1

CYTOLOGY OF NORMAL HUM

SYNOVIAL FLUID LEUKOCYTES

Percentage cell types

CASE	AGE	KNEE	AMOUNT	TOTAL LEUKOCYTES	TOTAL ERYTHROCYTES	POLYMORPHO- NUCLEARS	MONOCYTES	CLASMATO- CYTES	Unclassified phagocytes	Lymphocytes	Synovial cells	Unclassified cells	Total phagocytes	Polymorpho- nuclears
			cc.	Per c.mm.	Per c.mm.									
1	43	R	0.25	22	5	2	10	0	0	78	4	6	12	0.4
2	34	R	0.25	45	2000	6	24	6	10	51	3	0	46	2.7
	34	L	0.25	25	130	0	45	15	6	31	3	0	66	0
3	54	R	0.50	40	10	0	45	15	10	15	5	10	70	0
	54	L	0.50	54	51	4	50	26	8	8	4.3	0	88	2.2
4	75	R	0.25	55	15	6	61	0	0	15	12	6	67	3.4
	75	L	0.25	45	125	9	55	18	3	15	0	0	85	4
5	57	R	0.75	60	200	18	58	6	0	18	0	0	82	10.8
	57	L	0.50	30	20	4	70	6	6	14	0	0	81	1
6	80	R	0.25	75	15	4	34	22	10	24	6	0	70	3
	80	L	0.13	70	10	4	50	10	2	30	4	0	66	2.8
7	47	R	0.50	80	75	0	67	4	0	19	9	1	71	0
	47	L	0.50	65	+	6	55	21	9	9	0	0	91	3.9
8	15	R	0.25	50	15	0	45	12	4	39	0	0	61	0
	15	L	0.25	95	70	0	60	6	8	18	8	0	74	0
9	49	R	1.0	75	0	0	77	2	4	15	2	0	83	0
	49	L	2.0	90	0	0	54	8	0	32	6	0	62	0
10	56	L	0.38	60	0	24	46	6	0	22	2	0	76	14.4
11	64	R	0.38	85	5	0	68	0	2	22	6	2	70	0
	64	L	0.38	75	820	24	52	6	6	6	6	0	88	18
12	57	R	0.25	115	0	2	62	12	2	18	4	0	78	2.3
	57	L	0.25	75	0	0	56	24	0	14	6	0	80	0
13	61	R	0.75	180	50	2	30	21	8	34	3	2	61	3.6
14	55	R	0.50	13	0	18	40	3	12	12	10	5	73	2.3
	55	L	0.50	19	3	6	34	12	21	9	10	8	73	1.1
15	32	R	0.25	100	630	2	70	12	0	14	0	2	84	2.0
	32	L	0.75	65	15	4	68	16	2	6	2	2	90	2.6
16	47	R	0.2	20	0	25	0	0	0	65	0	10	25	5
	47	L	0.5	50	0	20	5	5	0	60	0	10	30	10
Maximal deviation	80		2.0	180	2000	25	77	26	21	78	12	10	91	18
Minimal deviation	15		0.13	13	0	0	0	0	0	6	0	0	12	0
Average	51		0.45	63.2	157.9	6.5	47.9	10.1	4.9	24.6	4.3	2.2	69.1	3.3

It will be noted that the sum of the average absolute counts is 63 instead of 63.2. This discrepancy is due to the fact that the figures were never carried beyond tenths.

SYNOVIAL FLUID (KNEE JOINTS)

C	SYNOVIAL FLUID LEUKOCYTES						BLOOD LEUKOCYTES		TERMINAL STATE			ANATOMICAL DIAGNOSES
	Absolute counts					DEAD CELLS SEEN IN COUNTING 100 NUCLEATED CELLS	Polymorphonuclears		Absolute count	Edema	Temperature	
	Unclassified phagocytes	Lymphocytes	Synovial cells	Unclassified cells	Total phagocytes		Total count	Per cent				
0	0	17.2	0.9	1.3	2.6	12				0	101°	Carcinoma of pancreas, cholecystgastrostomy and jejunostomy
2.7	4.5	23	1.3	0	20.7	9	9,100	68	6,188	0	100°	Coronary occlusion
2.7	1.5	8	0.7	0	16.5	21	9,100	68	6,188	0	100°	
5	4	6	2	4	28	0	7,400	78	6,372	0	100°	Syphilitic heart disease, aneurysm of aorta with rupture, syphilitic aortitis
1.4	4.3	4	2.2	0	47.5	18	7,400	78	6,372	0	100°	
0	0	8.4	6.6	3	36.9	18	8,900	65	7,721	0	102°	Coronary occlusion, pul- monary infarcts
3	1.3	7	0	0	38.3	6	8,900	65	7,721	0	102°	
3.6	0	10.8	0	0	49	4				0	100°	Carcinoma of pharynx, coronary occlusion
2	2	4	0	0	25.8	0				0	100°	
3.5	7.5	18	4.5	0	52.5	26	9,100			0	100°	Cerebral thrombosis and infarcts, hypertensive heart disease
7	1.4	21	2.8	0	46.2	10	9,100			0	100°	
3.2	0	15.2	7	1	56.8	3	8,600	65	5,590	0	101.8°	Brain tumor, post-opera- tive death
3.6	5.9	5.8	0	0	59.2	30	8,600	65	5,590	0	101.8°	
6	2	20	0	0	30.5	45	17,000			0	107°	Intracranial hemorrhage
5.8	7.6	17	7.6	0	70.3	20	17,000			0	107°	
1.5	3.0	11	0	0	62.3	0	10,400			0	99°	Spongioblastoma, cere- bral
7.2	0	28.8	5.4	0	55.8	8	10,400			0	98.4°	
3.6	0	13.2	1.7	0	45.6	4				0	102°	Pituitary tumor, post- operative death
0	1.7	18.7	5.1	1.7	59.5	10	10,000	90	9,000	0	102°	Meningioma, post-opera- tive death
4.5	4.5	4.5	4.5	0	66	8	10,000	90	9,000	0	102°	
3.8	2.3	20.7	4.5	0	89.7	2	33,700			0	105°	Carcinoma of stomach, gastric hemorrhage
8	0	10.5	4.5	0	60	10	33,700			0	105°	
7.8	14.4	61.2	5.4	3.6	109.8	0				0	108°	Trigeminal neuralgia, post-operative death
0.4	1.6	2.0	1.3	2	9.5	0	24,000	89	21,800	0	108°	Carcinoma of intestine with intestinal obstruc- tion
2.3	4.0	1.7	1.5	1.6	13.9	0	24,000	89	21,800	0	108°	
2	0	14	0	2	84	0	14,700	83	12,400	0	102°	Brain tumor, post en- cephalogram death
0.4	1.3	3.9	1.3	1.3	58.5	0	14,700	83	12,400	0	102°	
0	0	13	0	0	5	0				0	102.5°	Cerebral hemorrhage
2.5	0	30	0	0	15	0				0	102.5°	
7.8	14.4	61.2	7.6	4	109.8	45	33,700	90	21,800	0	108°	
0	0	1.7	0	0	2.6	0	7,400	65	5,590	0	98.4°	
6.7	2.6	14.7	2.5	0.8	45.4	9.8	13,900	76.9	9,864	0	102.4°	

were made of the undiluted fluid in a standardized hemocytometer. Differentiation of the nucleated cells from red blood corpuscles was facilitated if the counting chamber cover slip had been previously stained with cresyl blue or Unna's stain. The differential cell counts were made according to the supravital technique previously described (Warren, Bennett and Bauer, '35; Sabin, Doan and Cunningham, '24, '25; and Cunningham Sabin and Doan, '25). That the supravital technique greatly facilitates and allows for more accurate identification of the various types of mononuclear phagocytes than any fixed stain preparation is well illustrated in figure 1. Without the aid of the supravital technique, accurate differentiation is impossible.

RESULTS

Synovial fluid was aspirated from twenty-nine human knee joints in the manner described. In so far as could be determined, these individuals had never had joint disease and had never exhibited any evidence of edema or infection.

Such aspirated fluid from the normal human knee joint is clear, pale yellow to straw colored, extremely viscous and does not clot on standing. The most striking physical property is its marked viscosity. In the normal joint at rest the fluid is not under pressure and must be removed by aspiration. The results of this study as presented in table 1 reveal that the average quantity of synovial fluid obtainable from a human knee joint is 0.45 cc. with a minimal-maximal variation of 0.13 to 2.0 cc. Greater quantities of fluid are obtained only in the presence of edema or when a hypodermoclysis has been given in the region of the knee joint just prior to death. Fluids from such patients have not been included in this study.

As can be seen from table 1, normal human synovial fluid is relatively acellular, containing from 13 to 180 leukocytes per cubic millimeter, the average being 63 cells per cubic millimeter. The total cell counts on knee joint fluids from the same individual in most instances were approximately the same. That the variations in the total number of synovial fluid

leukocytes are due to other causes than fluctuations in the total leukocyte count of the blood is clearly shown in figure 2. Here one notes that case 14 had a total blood leukocyte count of 24,000 per cubic millimeter, whereas the knee joint fluids

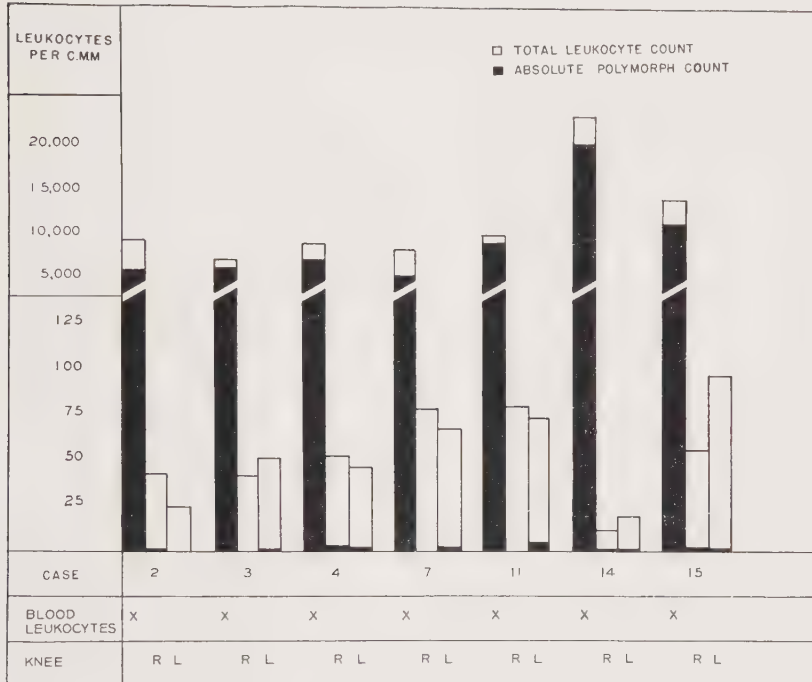


Fig. 2 This chart illustrates the marked differences in the total and absolute polymorphonuclear leukocyte counts of blood and synovial fluid of seven patients. Each set of columns represents the findings in the blood and in the synovial fluid from the two knees of the same patient, the two columns on the right representing the synovial fluid from the right and left knees. The numbers and letters below correspond with those given in table 1.

contained 12 and 13 leukocytes per cubic millimeter respectively.

Erythrocytes were absent in eight instances (table 1), thus indicating that they are not present in normal human synovial fluid. When found, they are undoubtedly the result of trauma incident to the aspiration.

Examination of table 1 reveals that the nucleated cells contained in normal synovial fluid have been divided into groups according to the classification of Sabin, Doan and Cunningham ('24 and '25) and Cunningham, Sabin and Doan ('25). Employing this classification, one can divide all nucleated cells into two major groups: 1, phagocytic, and 2, non-phagocytic. The cell types present are expressed in percentage figures as well as absolute counts (see table 1). The latter method has the advantage of portraying at once a more accurate cellular definition of any particular fluid. This is extremely helpful when dealing with fluids containing so few nucleated cells, in that the variations of individual cell types are not so great when recorded in this manner.

From table 1 it will be seen that the majority (69.1%) of the nucleated cells in normal synovial fluid belong to the phagocytic group, the average absolute count being 45.4. The mononuclear phagocytes (monocytes, clasmatoocytes and unclassified mononuclear phagocytes) comprise 63% of all synovial fluid cells, the average absolute count being 41.7. Of this group, monocytes are the predominant cell type, ranging from 0 to 77%, the average being 47.9%. The absolute number of monocytes varies from 0 to 71.3 per cubic millimeter, the average being 32.4 of the average total leukocyte count of 63 per cubic millimeter. Clasmatoocytes, absent in four fluids, reach 26% in one instance with an average of 10.1%, the average absolute count being 6.7. Unclassified mononuclear phagocytes range from 0 to 21%, averaging 4.9% or 2.6 cells. Polymorphonuclear leukocytes, absent in nine fluids, average 6.5%, with a maximum of 25%. Expressed in absolute counts, the greatest number of polymorphonuclear leukocytes per cubic millimeter is 18, with an average of 3.3 per cubic millimeter. The characteristic variations in phagocytic cells encountered in the normal synovial fluids studied are well illustrated in figure 3.

The non-phagocytic cells present in synovial fluid consist of lymphocytes, synovial cells and unidentified or primitive cells. The entire group represents 31% of all nucleated cells.

The lymphocytes are the predominant non-phagocytic cell, varying from 6 to 78%, the average being 24.6% or 14.7 per cubic millimeter. Synovial cells are never found in large numbers, the minimal-maximal percentage figures being 0 to 12%, with an average of 4.3%. Regardless of the method

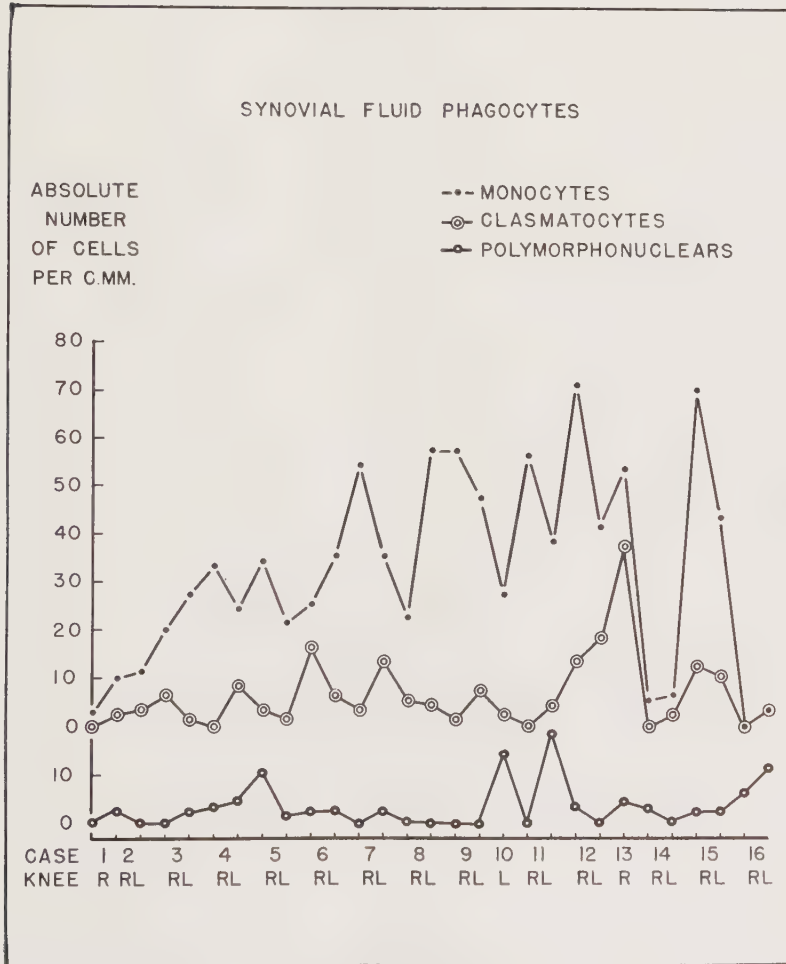


Fig. 3 This chart shows the absolute number of monocytes, clasmatoocytes and polymorphonuclear leukocytes per cubic millimeter in each normal synovial fluid examined. In order to prevent superimposing the lines representing clasmatoocytes and polymorphonuclear leukocytes, these two types of cells are charted separately.

employed, there always remain a few cells that cannot be identified.

Eosinophilic or basophilic polymorphonuclear leukocytes were not observed in any one of the twenty-nine synovial fluid specimens examined. Therefore, one must conclude that they are rarely present in normal synovial fluid.

DISCUSSION

From the data presented, it will be noted that the total nucleated cell count and percentage of individual cell types contained in normal human synovial fluid vary considerably from case to case. Despite these individual variations, it will be seen from figure 4 that the average total cell count in human fluid is much lower than in any animal species thus far studied. One also notes from figure 4 that there exists considerable species variation in the number of phagocytic and non-phagocytic cells contained in normal fluid. However, figures 4 and 5 both demonstrate that these individual cell type variations are much more marked in the non-phagocytic than in the phagocytic cell group. In fact figure 5 illustrates that although the total number of any one type of phagocytic cell may vary slightly from species to species (Warren, Bennett and Bauer, '35) the order of frequency of such cell types, from greatest to least, is always the same, namely, monocytes, elasmatoocytes and polymorphonuclear leukocytes. From the above it becomes apparent that the interpretation of the cytological characteristics of the various types of joint effusions encountered in the clinic must be based on a comparison with the findings in normal human synovial fluid.

The gross similarities of the cytological characteristics of the human fluids reported in this series and normal animal synovial fluids serve as supportative evidence that the twenty-nine human knee joint fluids examined were normal synovial fluid specimens. Further reason for believing that the studies herein reported represent the cytological characteristics of normal human synovial fluid is that all fluids examined were obtained from the knee joints of individuals without a history

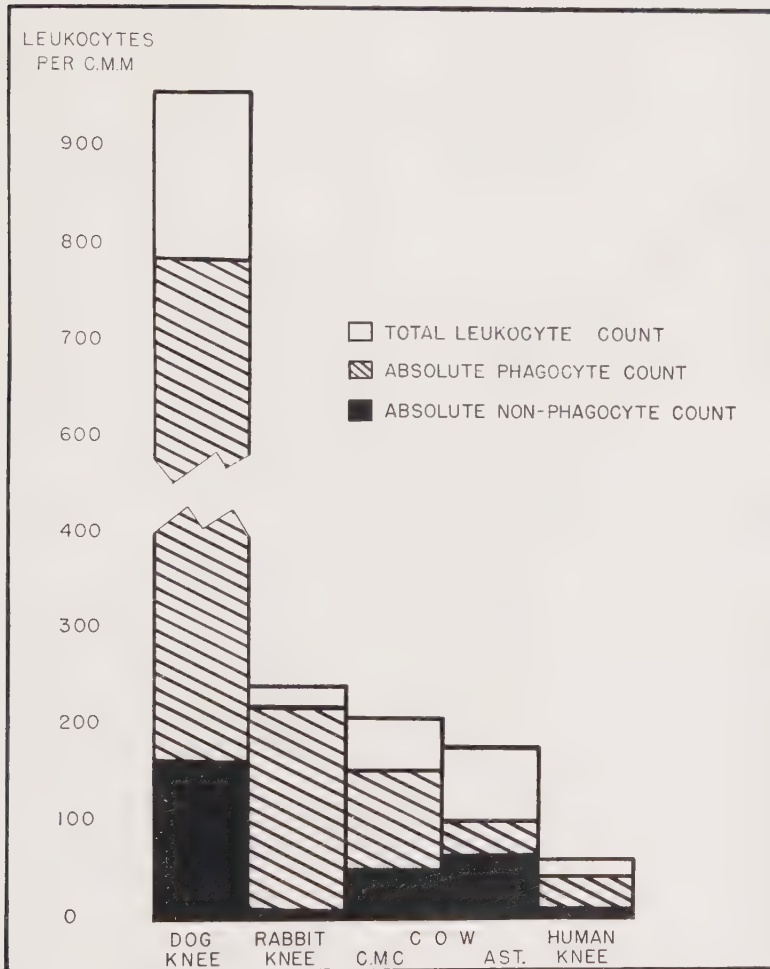


Fig. 4 The above chart shows the total number of leukocytes and the distribution of phagocytic and non-phagocytic cells in normal synovial fluid of the dog, rabbit and cow as compared with the human. Phagocytes include polymorphonuclear leukocytes, monocytes, clasmatocytes and unclassified mononuclear phagocytes. The columns are read from the base line at zero. In the case of the cow, the fluid from two joints was examined, namely, C.M.C. or the carpometacarpal and AST. or the astragalotibial joints.

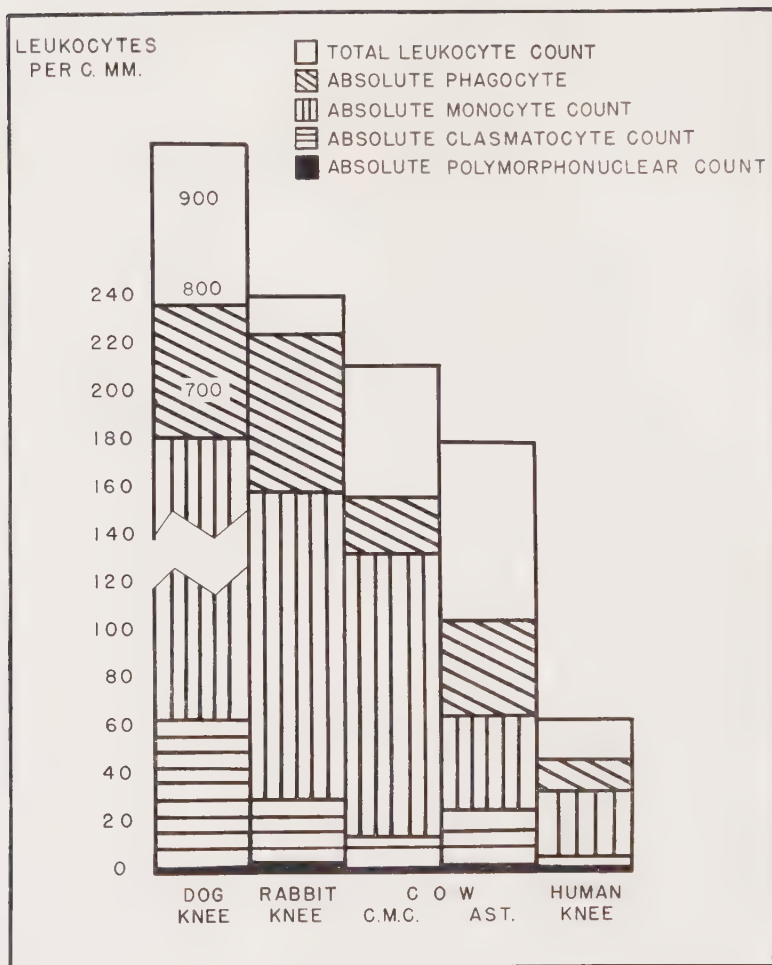


Fig. 5 The above chart shows the distribution of the various types of phagocytes in normal synovial fluid of animals as compared with the human. Each column and its components are read with zero as the base line. The first column representing the phagocytes contained in the knee fluid of dogs has a separate set of numbers with a scale from zero to 900 per cubic millimeter. Again the abbreviations C.M.C. and AST. are used to signify the carpometacarpal and astragalotibial joints.

of previous joint disease and not exhibiting signs of infection or edema at the time of death. In this and previous studies (Warren, Bennett and Bauer, '35), it has been shown that factors such as the total number of circulating leukocytes and post mortem migration of cells from the subsynovial tissue or capillaries into the joint fluid do not alter the synovial fluid cytology. That contamination of synovial fluid by blood at the time of aspiration is not an important influencing factor is shown by the fact that the cytology of the eight fluids containing no erythrocytes is not materially different from those in which a few erythrocytes were introduced at the time of aspiration. Having excluded all the above-mentioned influencing factors, we are of the opinion that the results obtained in the present study represent the cytological variations encountered in normal human synovial fluid.

From the results obtained in these studies it is apparent that normal synovial fluid is a relatively acellular fluid. This fact is not surprising when one appreciates that normal synovial fluid serves chiefly as a lubricant for the articulating surfaces and as a source of nourishment for articular cartilage. The predominant cell type is the mononuclear phagocyte. From previous studies, it would appear that its chief function is the removal of the wear and tear products resulting from minor traumata and insults (Bauer, Bennett, Marble and Claffin, '30; Warren, Bennett and Bauer, '35). That type and degree of trauma influence synovial fluid cytology can readily be demonstrated by injecting saline into a normal rabbit's knee joint (Warren, Bennett and Bauer, '35). Such mild irritation results in an immediate and sudden rise in the polymorphonuclear leukocytes which are replaced subsequently by a corresponding rise of mononuclear phagocytes. Apparently such mild irritation produces sufficient change in the subsynovial capillaries to allow for an immediate intra-articular migration of polymorphonuclear leukocytes, whereas the mononuclear phagocytes arising from the subsynovial tissue and tissue fluid are much slower to respond. Therefore, we are of the opinion that the variations in total cells and

individual cell types encountered in normal human synovial fluid probably represent the intra-articular cytological response to the inconstant traumata incident to daily use.

As can be seen from table 1, the fluids examined were obtained from individuals of varying ages. This series is too small to attempt to demonstrate any correlation between the cellular variations and increasing age. However, from previous studies it was learned that with increasing age one notes increasing evidence of degenerative joint changes (Bennett and Bauer, '33 and unpublished data). These changes are noted in the knee joints of some normal individuals (without any history of previous joint signs or symptoms) in the third decade and in all individuals by the time they reach the fifth decade. These changes, indistinguishable from those commonly considered characteristic of so called hypertrophic arthritis or degenerative joint disease, are thought to represent chiefly the wear and tear changes of increasing age and long-continued use in a tissue (articular cartilage) which has a very limited ability to repair itself. That the cytological variations noted are probably related to these changes is suggested by the fact that the three joints (examined at autopsy) showing the greatest degree of such degenerative changes had the highest total cell counts. This and the fact that the majority of the synovial fluid leukocytes are mononuclear phagocytes tend to corroborate our previous interpretation that the leukocytes under normal conditions are chiefly concerned with the removal of the debris and particulate matter resulting from the wear and tear changes incident to daily use. The total cell count in many instances might have been higher if the joints had been subjected to normal use up to the time of death.

From the above-mentioned findings one might predict that synovial fluid from a joint with uncomplicated degenerative joint disease (commonly called hypertrophic arthritis) would probably show the same cell type variation encountered in normal synovial fluid. However, one might expect the total cell count to be higher. That such is the case will be shown in subsequent studies (unpublished data).

If, as some workers contend (Collins, '36; Hueck, '20; Jones, '30; King, '35 and unpublished data), the joint cavity is merely an enlarged tissue space lined by specialized connective tissue, then the cytology of synovial fluid may be considered to be essentially similar to that of tissue fluid.

SUMMARY

From a study of synovial fluid aspirated from twenty-nine normal human knee joints, at the time of death, the following conclusions can be made:

1. Normal human synovial fluid is clear, pale yellow to straw colored, extremely viscous and does not clot on standing.

2. The average amount of such fluid obtainable by aspiration is 0.45 cc. The minimal-maximal variations are 0.13 to 2.0 cc.

3. The average total nucleated cell count per cubic millimeter is 63 cells. The variations range from 13 to 180 cells per cubic millimeter.

4. Based on an average synovial cell count of 63 cells per cubic millimeter, the percentage and absolute number of cell types are: 63% or 41.7 mononuclear phagocytes, 24.6% or 14.7 lymphocytes, 6.5% or 3.3 polymorphonuclear leukocytes, 4.3% or 2.5 synovial cells and 2.2% or 0.8 unidentified cells.

5. The function of the synovial fluid leukocytes is the removal of the wear and tear debris incident to daily use.

6. The variations in total and individual cell type counts are probably dependent upon the inconstant tissue trauma resulting from the daily insults incurred with joint use.

7. It is suggested that the cytology of synovial fluid is essentially similar to that of tissue fluid.

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CADAVER ENCEPHALOGRAPHY WITH OPAQUE CONTRAST MEDIUM

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ONE TEXT FIGURE AND TWO PLATES (TWO FIGURES)

INTRODUCTION AND PROCEDURE

An exact understanding of normal anatomical details in encephalograms is not only of practical importance for the clinician but also of interest to the anatomist. The procedure of encephalography has been fittingly described as a method of anatomy in vivo. The demonstration of structures and contours with the help of air as a contrast medium, however, often gives only vague pictures, the interpretation of which may cause considerable difficulty.

Several years ago the author undertook a series of correlated anatomical and clinical studies on encephalography with the collaboration of Dr. O. Hahn of the Department of Surgery of the University of Breslau. In order to obtain a set of pictures with more contrasting and sharply defined outlines, a number of fresh cadavers were given injections of about 40 to 60 cc. of a suspension of barium sulfate directly into the lateral ventricle following the routine procedure for ventricular puncture. To facilitate interpretation the method of stereoscopic roentgenography was used. Our encephalographic studies were interrupted and remained unpublished with the exception of a brief report on a clinical case ('30).

In the meantime L. M. Davidoff and C. G. Dyke published a series of papers on the demonstration of normal anatomical structures by means of encephalography and presented an interesting summary of their results in book form ('37). The study of this book has prompted the author to present the

following brief description of some of his results obtained by cadaver encephalography with opaque medium.

OBSERVATIONS

Figure 2 shows an antero-posterior view. The lateral ventricles are well outlined. The interventricular foramen is visible on the right side and overlapped by the anterior horn on the left, the third ventricle is clearly defined. The most interesting feature of the ventricular system in this view, however, is the unusual aspect of the fourth ventricle which gives a shadow comparable to an inverted butterfly figure. The tips of the wings directed laterad and caudad correspond to the superior posterior recesses of Retzius. The lighter space between the smaller wings directed upward corresponds to a lesser filling of the ventricle due to the basal convexity of the velum medullare anterius and the lingula cerebelli protruding into the ventricle. The aqueduct of Sylvius is obscured by the shadow of the cisterna ambiens which is well filled and extends laterally to the inner aspect of the temporal lobes, adjoining the temporal horns of the lateral ventricles. A small amount of contrast material is seen in the foreshortened longitudinal fissure which is partly covered by the shadow of the injection needle entering the left lateral ventricle. The left cerebellar hemisphere is outlined, the contrast medium has also penetrated into some of the cerebellar sulci, showing the sulcus superior, sulcus horizontalis cerebelli and sulcus inferior posterior, so that the superior and the inferior semilunar lobules are visualized dorsally and basally to the horizontal sulcus. Within the posterior incisure of the cerebellum the right cerebellar tonsil can be seen. The cisterna magna is shown with its continuity into the cervical spinal subarachnoid space where the outline of the spinal cord is clearly visible, presenting a very conspicuous intumescencia cervicalis.

Figure 3 represents the lateral (dextro-sinistral) view of the same case. It is of interest to compare this view with a mid-sagittal section through the head which has been similarly

labeled (fig. 1). The lateral ventricles show obliterated posterior horns, the right lateral ventricle has also an obliterated temporal horn, findings which were confirmed by the subsequent dissection. The third ventricle and the interventricular foramina appear, a great part of the third ventricle is overlapped by the cisterna ambiens and interpeduncularis. The caudal part of the aqueduct of Sylvius is brought out together

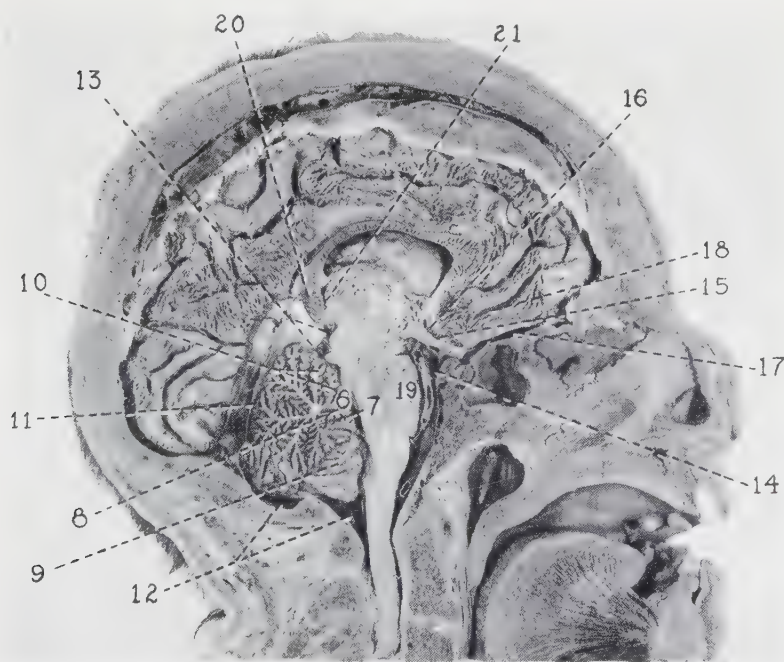


Fig.1 Midsagittal section of an adult head showing topographic relations of cerebral structures. Labels same as in figure 3.

with the fourth ventricle showing the fastigium which is bounded caudally by the nodulus of the cerebellum. Adjacent to the nodulus the caudal part of the choroid plexus of the fourth ventricle is visualized.

The outline of the cerebellum forms a quite conspicuous feature. Behind the nodulus the uvula can be recognized and the tonsilla stands out projecting into the cisterna cerebello-

medullaris. Rostrad the monticulus and lobulus centralis are outlined by filling penetrating from the cisterna ambiens. The general contour of the cerebellum is furthermore indicated by a thin layer of contrast material following the concavity of the tentorium. In various places individual folia may be visualized due to the penetration of the material between them.

The filling of the subarachnoid spaces shows the outlines of spinal cord, oblongata and pons. The cervical intumescence of the spinal cord appears also in this view, furthermore a number of roots on the left side, the dorsal roots of the fourth cervical nerve and the dorsal and ventral roots of the fifth and sixth cervical nerves.

Further rostrad and dorsad the splenium of the corpus callosum is visualized, basal to it the suprapineal recess beneath which the pineal body can be recognized. Several of the cisternae are well filled and appear very distinctly, besides the cisterna magna already mentioned the cisternae pontis, ambiens, interpeduncularis, chiasmatis and laminae terminalis cinereae are shown.¹ Within this latter cistern the anterior communicating artery appears quite distinctly. In this specimen an injected subarachnoid space extends rostrad from the cisterna chiasmatis at the base of the frontal lobe along the olfactory tracts, forming, as it were, a cisterna olfactoria. Dorsal to this structure the rostral sulci have become visible on the medial surfaces of the hemispheres.

DISCUSSION

While almost all of the structures mentioned have been demonstrated in the living by means of encephalography with air or other gases as contrasting media, the cadaver encephalography with opaque medium shows usually more distinct outlines and frequently gives a simultaneous picture of a greater

¹ The negative showed in the interpeduncular cistern a faint but sufficiently clear outline of the oculomotor nerve and of the tuber cinereum with the hypophyseal stalk as well as an outline of the optic nerve and chiasma in the cisterna chiasmatis. These details, however, do not appear distinctly in the reproduction.

number of different structures, so that its usefulness as a supplementary method for the study of normal intracranial topography in the roentgenogram seems well established.

The peculiar aspect of the fourth ventricle in antero-posterior view, the visualization of an olfactory cistern, the delineation of the intumescencia cervicalis and the visualization of cervical nerve roots should be especially mentioned as probably new or at least very unusual features.

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PLATE 1

EXPLANATION OF FIGURE

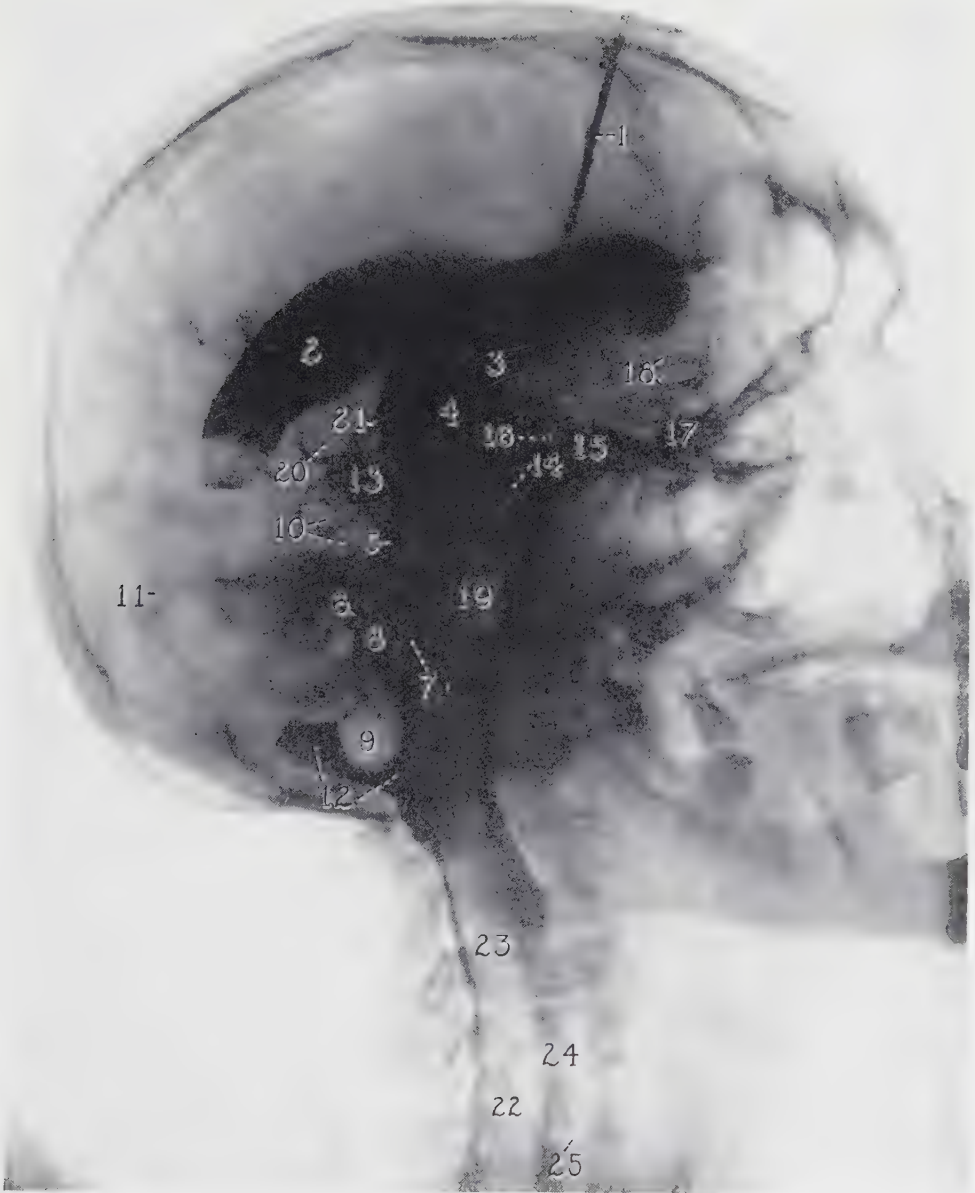
2 Antero-posterior view. 1, injection needle; 2, longitudinal fissure; 3, lateral ventricle; 4, foramen interventriculare; 5, third ventricle; 6, fourth ventricle; 7, fourth ventricle; recessus superior posterior; 8, cisterna ambiens; 9, cisterna magna; 10, incisura cerebelli posterior; 11, tonsilla cerebelli; 12, left cerebellar hemisphere, lobulus semilunaris superior; 13, sulcus horizontalis cerebelli; 14, intumescencia cervicalis.



PLATE 2

EXPLANATION OF FIGURE

3 Lateral view (dextro sinistral). 1, injection needle in left lateral ventricle; 2, right lateral ventricle with obliterated posterior and temporal horns; 3, inter-ventricular foramen; 4, third ventricle; 5, aqueduct; 6, fourth ventricle, fastigium; 7, choroid plexus of fourth ventricle; 8, nodulus cerebelli; 9, tonsilla cerebelli; 10, monticulus and lobulus centralis; 11, outline of tentorium; 12, cisterna magna; 13, cisterna ambiens; 14, cisterna interpeduncularis; 15, cisterna chiasmatis; 16, arteria communicans ant. in cisterna laminae terminalis; 17, cisterna olfactoria; 18, sulci rostrales; 19, pons; 20, splenium corporis callosi; 21, recessus suprapinealis; 22, intumescencia cervicalis; 23, dorsal roots of fourth cervical nerve; 24, roots of fifth cervical nerve; 25, roots of sixth cervical nerve.



THE EFFECT OF THE OVARY AND OF URINARY ESTROGENS ON THE GROWTH OF HAIR IN THE RABBIT

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That the sex glands play a rôle in controlling the growth of hair is suggested by the sexual dimorphism in the pilary system of man. The appearance of increased amounts of terminal, or definitive hair at the age of puberty also points to the gonads as factors regulating the growth of hair. The early observations of Tandler and Grosz ('09, '10) on the complete disappearance of the beard, and on the changes in the distribution of hair following post puberal castration is further evidence of a similar nature.

Among the experimental studies of Steinach ('12, '13), there is reference to alteration of the character of the hair following the transplantation of ovaries into castrated male guinea pigs. But, as pointed out by Cooper ('30), there is still no consensus of opinion as to the relative importance of the various organs of internal secretion with respect to their effect on the pilary system.

In the course of another experiment (Frazier, Mu and Hu, '35) we observed that the regrowth of hair following shaving in rabbits treated with urinary estrogens differed from that in normal animals. In this connection, Steinach, Heilein and Wiesner ('25) found that the injection of ovarian and placental extracts into senile female rats resulted in regrowth of hair over previously bald spots, and that the entire coat of the treated animals became thick and glossy. Analogous to these results, however paradoxical it may seem at first view, Peters ('34) met with success in treating hirsutism with oestril,

an estrogenic substance, inasmuch as hairs fell for a number of days after each of a series of treatments. Barbour, in discussing the report of Peters, described the regrowth of hair over the vertex of the scalp of a woman who presented the masculine type of alopecia together with facial hirsutism, but apparently estrogenic (theelin) treatment was without any effect on the growth of facial hair. In both the experimental and clinical observations, the action of the sexual hormones was to reestablish, at least partially, a normal feminine condition.

As opposed to these results, Dawson ('33) who studied this problem systematically, found in guinea pigs that the average rate at which hair grew was higher in the male than in the female, and that in breeding females, the average rate decreased during pregnancy, reaching its minimum soon after delivery.

For a final proof that the sex glands control hair growth in some way, conclusive evidence is still to be adduced. Obviously, such evidence is to be obtained best through subjecting laboratory animals to tests, where the variable factors may be adequately controlled under specially designed experimental conditions.

During the past 3 years we have carried out a number of experiments designed to determine the influence of sex hormones on the regrowth of hair following shaving in the rabbit. The results we have obtained thus far are presented below. These concern in particular: 1. Age; when the sex glands were functioning, and when not. 2. Sex; the difference normally existing between the two sexes. 3. Feminization of males through estrinization; where the testicles atrophied. 4. Ovariectomy; where the female sex hormone producing organ was removed.

MATERIAL AND METHOD

Chinese albino rabbits of both sexes were used. Their age varied between $2\frac{1}{2}$ and 20 months. In many cases, litter mates of different or the same sex were employed. The upper part

of the abdomen was closely shaved with a razor. Observations were made at intervals of a week. As soon as hairs started to regrow, one small area was stained blackish with hair dye (paraphenyldiamine). Two weeks were allowed to elapse, and the stained area was reshaved. The stained portion, representing the length of growth during the period between shaving and staining, was measured against a steel ruler which was graded to 1 mm. and read to 0.5 mm. Twenty hairs of each shave were measured. This number was found to be adequate for statistical computations. The shaved area was usually divided into six parts, and one of these stained each consecutive week until growth stopped, as indicated by the failure in appearance of a whitish portion at the proximal part of the shaved hair.

Repeated shaving. A number of animals were shaved two or three times in order to see whether shaving alone exerts any effect on the growth of hair.

Ovariectomy. Ovariectomy was performed on animals 3–5 months of age. Both ovaries together with the surrounding fat and part of the fallopian tubes were removed while the animal was under ether anesthesia. Shaving was not done until $1\frac{1}{2}$ – $3\frac{1}{2}$ months after the operation.

Estrinization. Estrinization of male animals was effected through subcutaneous injections of the ether soluble fraction of a butyl alcohol extract of urine of pregnant women (Dawson, '33) administered in dosages of 30–60 rat units (equivalent to approximately 100–200 I.U. of estrone) daily, 6 days a week. The abdomen was shaved after $4\frac{1}{2}$ months of estrinization, when manifest effects were shown in the feminization of the male genital organs (Frazier and Mu, '35), enlargement of the nipples and the presence of dewlap (Hu and Frazier, '38).

RESULTS

The results are summarized in table 1. It will be seen that a number of factors came into play either singly or in combination. These factors were: age, sex, ovariectomy, and estrinization. The influence of each of these factors on the

regrowth of hair after shaving is separately analyzed. The influence is measured by: (1) the time of reappearance of hair, (2) the time required for a full regrowth in length, and (3) the rate of regrowth during the whole period from onset of regrowth to full growth, or any portion thereof. Of the three observations, the onset of regrowth was the most clear cut

TABLE 1

General survey. Influence of age, sex, ovariectomy and estrinization on the onset of regrowth, time of full regrowth and rate of regrowth of hair following shaving

GROUPS ¹	ONSET OF REGROWTH	FULL REGROWTH	RATE OF REGROWTH
	<i>weeks</i>	<i>weeks</i>	<i>mm. per week</i>
I. Immature, normal (2½ mos. old)			
A. Males (7) } litter mates	4.7±0.22	6.4±0.42	3.8±0.55
B. Females (4) }	4.5±0.47	6.0±0.70	8.9±0.86
II. Borderline mature, normal (4½-6 mos. old)			
A. Males (6) litter mate pairs	3.6±0.44	7.5±0.53	6.3±1.03
B. Females (8) litter mate pairs	1.6±0.14	5.5±0.28	3.4±0.32
III. Fully mature males, normal (8) ² (20 mos. old)	3.4±0.07	5.0±0.00	4.8±0.42
IV. Estrinized and normal males (1 yr. old)			
A. Normal males (6) } litter mates	3.0±0.00	5.2±0.14	5.8±0.44
B. Estrinized males (7) }	1.3±0.05	5.4±0.09	2.7±0.19
V. Ovariectomized and normal females (7-8 mos. old)			
A. Normal females (8) } litter mates	1.8±0.19	5.7±0.35	4.7±0.33
B. Ovariectomized (5) }	5.2±0.83	7.4±0.61	5.1±0.72

¹ Simple numerals in parentheses indicate number of animals employed.

² Six other animals showed no sign of regrowth in 9 weeks, and are not included in the table.

NOTE: All the tables deal with mean values, and the significance of the mean is indicated by standard deviation of the mean instead of its probable error. In all the tables the term "onset of regrowth" refers to the interval of time between shaving and reappearance of hair.

and easiest to measure. For reasons to be discussed later, the results of the other two measurements were inconclusive, and therefore not reported here in detail.

Age. In determining the influence of the age factor, the factor of sex was excluded by comparing only the results within the same sex groups. Those animals which were ovariectomized or estrinized were also excluded from consideration.

The animals were divided into three age groups, namely, immature, borderline mature, and fully mature, with age limits for the respective groups as indicated in table 2. Table 3 shows: (1) In the case of normal males, the hairs reappeared significantly earlier in the fully mature than in the immature animals ($P = 0.001 -$), while the borderline mature and immature groups showed a similar and significant difference ($P = 0.001 -$). (2) In the case of normal females, the differences were similar to those obtained for the males. Both the fully

TABLE 2

The influence of age and of sex on onset of regrowth of hair following shaving

AGE GROUP	ONSET OF REGROWTH IN WEEKS ¹	
	Male	Female
1. Immature ($2\frac{1}{2}$ mos.)	4.7 ± 0.22 (7)	4.5 ± 0.47 (4)
2. Borderline mature ($4\frac{1}{2}$ -6 mos.)	3.6 ± 0.44 (6)	1.6 ± 0.14 (8)
3. Fully mature (7-20 mos.)	3.2 ± 0.04 (14)	1.8 ± 0.19 (8)

¹ Numerals in parentheses indicate number of animals employed for observation.

TABLE 3

Test for significance¹ of results listed in table 2

P VALUES FOR COMPARISON BETWEEN SEXES		P VALUES FOR COMPARISON BETWEEN AGE GROUPS		
Age groups	Male vs. Female	Age groups ²	Male	Female
1. Immature	0.484	1 vs. 2	0.014	0.001 -
2. Borderline mature	0.001 -	1 vs. 3	0.001 -	0.001 -
3. Fully mature	0.001 -	2 vs. 3	0.159	0.468

¹ In tables 3, 4, and 5, Fisher's method for testing the significance of results obtained from small samples was used.

² Comparison between age groups as listed in the first column on the left hand.

mature and the borderline mature females showed a significantly earlier regrowth than did the immature females (the values of P for the first two groups as compared with the immature group being the same, $0.001 -$). However, the difference between the borderline mature and the fully mature groups was not statistically significant for either sex (P for male, 0.159; and for female, 0.468).

Sex. Table 3 also presents figures showing the influence of sex on the reappearance time of hair after shaving. In the

immature group of animals, the males and females showed no significant difference ($P = 0.484$). In the borderline mature group, the females grew hairs much sooner than did the males, this difference being statistically significant ($P = 0.001 -$). Likewise, in the fully mature group, the females grew hairs much sooner than did the males ($P = 0.001 -$). Furthermore, the regrowth in the males was frequently irregular and incomplete for weeks. In six of a group of fourteen mature males, there was no appreciable regrowth of hair during a period of 9 weeks after shaving (see footnote, table 1, group III).

TABLE 4
Influence of estrinization of males on onset of regrowth

GROUP		ONSET OF REGROWTH IN WEEKS	P VALUE
Normal males (6)	} Litter mates	3.0 ± 0.00	0.001 -
Estrinized males (7)		1.3 ± 0.05	

TABLE 5
Influence of ovariectomy on onset of regrowth

GROUP		ONSET OF REGROWTH IN WEEKS	P VALUE
Normal females (8)	} Litter mates	1.8 ± 0.19	0.001 -
Ovariectomized (5)		5.2 ± 0.83	

Estrinization. A total of thirteen male rabbits about 1 year of age were used for the experiment, six were left untreated and served as controls and seven were estrinized, the control and test animals being litter-mate pairs. Table 4 shows that the estrinized males had a regrowth of hair much earlier than did the controls. This difference is highly significant, $P = 0.001 -$.

Ovariectomy. The influence of ovariectomy on the regrowth of hair was marked, as may be seen from table 5. The mean time of the first appearance of hair after shaving for eight normal mature females was 1.8 weeks and that for five ovariectomized litter mates was 5.2 weeks. This difference is highly significant, $P = 0.001 -$.

Repeated shaving. In a group of ten mature females (three ovariectomized and seven with ovaries intact) shaving of the upper abdomen was repeated once or twice. Analysis of the results revealed no difference between the first and second or third shave, either in the time of reappearance of growth or in the rate of regrowth.

DISCUSSION

As may be seen from table 1, we have attempted to make observations on three points: (1) the onset of regrowth, (2) the time required to gain the full length of regrowth, and (3) the rate of regrowth during the period of observation. It was rather easy to mark the date of reappearance of hair, and with a fair degree of accuracy. But, it was difficult to determine the time when full regrowth was established. As a matter of fact, it was assumed that full regrowth was reached when the hairs seemed to have stopped growing further during the latter part of the observation period. The allowance of 2 weeks after staining might not have been sufficient to determine the point of full growth. Not infrequently the observations were terminated before the hairs actually reached the full limit of growth. Furthermore, there were other factors which contributed to make the end point not clear cut with respect to the time required both for full regrowth and for determining the rate of regrowth. Variations even between litter mates of the same sex were great. Hairs within the same area did not grow at the same rate, due perhaps, to the presence of more than one type of hair (Dawson, '30). As observed by Trotter ('24), the life cycle of hair may be divided into an active and a resting phase, and that at any one moment, all hairs are not in an active growing state.

For the reasons mentioned above, the measurements for full regrowth and for rate of regrowth showed great variations, and dependable conclusions, therefore, cannot be drawn from the results obtained thus far. Further observations on these points are desirable. But as far as statistical tests on the results go, the rate of regrowth differed significantly between

the two sexes in the immature group, and between the estrinized males and their litter-mate controls. In both the borderline mature and the fully mature groups there was no significant sexual variation, and the ovariectomized animals showed no difference of significance from their litter-mate controls, although in all of the three comparative groups there was a highly significant difference as far as the onset of regrowth was concerned. These inconsistencies could not be explained. Therefore, in this report, the results were analyzed with reference only to the onset of regrowth. The experimental errors in all cases were minimized by taking mean values of twenty hairs from each shave, by comparing values obtained for groups of animals instead of individuals, and by the use of litter mates as far as was possible.

Our observations on the effect of repeated shaving were few in number, but as far as they went, the negative results were confirmatory of Trotter's findings ('28).

It is noteworthy that in the immature group, males and females showed no significant difference in the time of the first appearance of hair following shaving. This could be explained on the ground that at this immature age ($2\frac{1}{2}$ months) the gonads are not yet functioning with respect either to reproduction or to internal secretion. However, normal mature males and females showed a decided difference in time of onset of regrowth.

The extirpation of the ovaries, before the animals reached full maturity, retarded the regrowth of hair. Although the actual figures given in table 2 deal with only five ovariectomized animals, observations on ovariectomized rabbits employed in other experiments fully supported the results obtained here.

When males were feminized through estrinization, the behavior of hair regrowth was brought close to that of the females. The change appeared to be clear cut.

It is to be emphasized that all the above results with respect to the onset of regrowth consistently pointed in the same direction. And, putting the findings together, only one con-

clusion is apparent, namely, that female sex hormones exert a definite stimulating influence on the onset of regrowth of hair after shaving, either directly or indirectly. The mechanism of action remains to be determined.

SUMMARY AND CONCLUSION

The results of our observations on the regrowth of hair in the rabbit after shaving may be stated as follows. Before the gonads function (immaturity) the time of reappearance of hair was about the same in males as in females. In the mature animal, under the natural influence of the respective sex hormone, the regrowth of hair appeared much earlier in the female than in the male. Upon removal of the female sex hormone producing organ (ovariectomy), the reappearance of hair was considerably delayed. Finally, when males were feminized through estrinization, the onset of regrowth of hair was hastened, approaching the female fashion. Thus, the conclusion is drawn that the ovary as well as urinary estrogens clearly exert a stimulating influence on the growth of hair in the rabbit, after shaving, at least as far as the time of its reappearance is concerned. The observations on the time of full growth and also on the rate of regrowth gave inconsistent results.

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VARIATION IN MOUSE EMBRYOS OF 8 DAYS GESTATION

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TWO PLATES (FIFTEEN FIGURES)

INTRODUCTION

In the mouse the primitive streak generally appears near the close of the seventh day of gestation (Sobotta, '11; MacDowell, Allen and MacDowell, '27). The eighth is consequently the first day in the history of the embryo proper. This paper records data on the variation in size and number of somites at the end of the eighth day.

When weighing embryos of triple hybrid mice for the study of prenatal growth (MacDowell, Allen and MacDowell, '27), individuals of only two 8-day litters were weighed separately. The other 8-day litters were weighed in groups. The variation in litter weight is shown in figure 1 of the paper referred to. The average weight in grams of eleven litters consisting of ninety-three embryos was 0.00008. In one of the litters weighed by individuals, the weights in grams varied from 0.00002 to 0.00025; in the other litter, from 0.00005 to 0.0004. In the present paper are shown photographs of several litters of 8-day Bagg-albino mice, which confirm the possibility of wide variation in mice embryos at this age. To how great an extent the early differences in size and degree of development may be compensated for in the later developmental processes is at present conjectural. On account of its relatively small size the mouse embryo offers excellent opportunity for study of the histology and cytology of the various organs, yet much remains to be discovered of these conditions at various stages in the history.

MATERIAL AND METHODS

The material for the data recorded in this paper came from the MacDowell colony at the Department of Genetics of the Carnegie Institution of Washington, at Cold Spring Harbor, New York. The data for the number of somites are from observations made on the triple hybrid mice employed in the study of prenatal growth (MacDowell, Allen and MacDowell, '27). The photographs are of white mice of the Bagg-albino strain. All the embryos were timed within an hour of insemination. Two males were placed with six females. At the expiration of an hour the males were removed and the females examined. Those which showed vaginal plugs were isolated in separate cages until killed for embryos. The time of sacrifice was based on the end of the hour during which the males and females were together.

The method of preparing the embryos for photographing was to kill the mother by ether or by breaking the neck by quick pressure with the operator's thumb on the cervical region. The uteri were immediately removed to Locke's solution in a low Stender dish, the bottom of which was covered with black wax. Under a low-power binocular microscope the uterine horns were turned ventral side up, stretched slightly, and pinned to the wax with the mesometrium flattened. The uterine muscles on the exposed surface were then carefully cut away from the decidual capsules. A shallow incision was made in the capsule wall to help admit the fixative, a small amount of which was immediately applied directly to the capsules by pipette. Then, by aid of needles and iridectomy scissors, the exposed side of the capsule was dissected away, additional fixative being pipetted on during the dissection (fig. 5). When the embryo was uncovered the mixture of Locke's solution and fixative was replaced by pure fixative. In some cases the photographs were taken before the fixative was finally added. In others, if Bouin's or B-15 fixative had been used, the uteri were left about 1 hour in the fixative and then run up to 50% alcohol, after which the dissection was completed. The decidual tissue dissects more easily in 50% than

in 70% alcohol. After photographing at the desired magnification the uteri, with the embryos in situ, were stored in separate dishes, or the embryos removed for sectioning.

The photographs chosen for reproduction represent only a few of the litters which showed marked variation. A litter at $8\frac{1}{2}$ days gestation is introduced for comparison in size (fig. 6).

It is difficult to fix and dehydrate embryos of this age without some distortion. Of those reproduced, figures 4 to 6 and 11 to 15 show the least—practically none. The gradual addition

TABLE 1

Variation in number of somites in ninety-five embryos in nine litters of triple hybrid mice at end of 8 days of gestation

LITTER NO.	NUMBER OF EMBRYOS IN LITTER	NUMBER OF SOMITES PER EMBRYO									DEGENERATE EMBRYOS
		0	1	2	3	4	5	6	7	8	
8-2	8	8									
8-3	10			2			2	5		1	
8-4	11	1	1	2	1	5	1				
8-5	10	6	1	2							1
8-6	10	2	1			2	4	1			
8-7	9			1		2	1	2			3
8-8	14	8	4	1	1						
8-9	12	1	2	2	1	2	1	2			1
8-10	11	5	1		1	2	1				1
9	95	31	10	10	4	13	10	10		1	6

of fixative to Locke's solution as described above usually results in less distortion than if the embryo is completely uncovered in Locke's solution, removed from the capsule, and then placed in the fixative.

The method used for the triple hybrid embryos recorded in table 1 was to take one capsule at a time from the uterus and remove the embryo under Locke's solution as described in the paper by MacDowell, Allen and MacDowell ('27). Meanwhile the uterus remained in the body of the mother until all embryos had been removed. Somite determination was made at

once under a binocular dissecting microscope while the embryo was still alive.

For purposes of record, the embryos were numbered by position in the uterine horn. Thus R-1 refers to the embryo nearest the vagina in the right horn. L-2, to the second embryo in the left horn.

OBSERVATIONS

In some litters the embryos in both horns of the uterus, whether equal in number or not, were about the same size. In some, as in figure 5, they were larger in one horn. In others, variations were almost the same in each horn (figs. 1 and 2). Position in horn, number in horn, or number in litter seems to have no influence on size or degree of development.

In the litter shown in figure 5, variation ranges from the primitive groove stage (R-2) to the 4-somite stage (L-1 and R-5); the other embryos vary between these two extremes. R-4 is in about the same stage as the two embryos shown enlarged in figure 7—early head fold. L-2 and L-3 are a little farther along than R-4, but with no somites. L-2 is shown enlarged in figure 8. R-3 and R-6 are slightly farther advanced than R-4, but not so far advanced as R-5 and L-1.

In figures 1-3, no embryo has reached the somite stage. Among the Baggs-albino strain, none at this age was found with more than three somites. The somite variation in the triple hybrid stock is from one to eight, based on ninety-five embryos (table 1). Of the normal embryos, thirty-one had no somites; their development varied from the primitive groove to the head-fold stage. Only one embryo had eight somites. The number of embryos is too small to establish a mean number of somites.

No other data concerning stage of development are considered in this paper. The photographs indicate that gross size is an approximate index of somite number. By the end of another 12 hours ($8\frac{1}{2}$ days of gestation), the somites may vary from five to fourteen (one litter).

A type of variation was found in which one capsule might contain more than one embryo, each with its own yolk sac

(fig. 3, L-8 and 9, and fig. 13). Generally in such cases one embryo was smaller than the other. The two shown in figure 13 were the nearest in size of any found at this age. Among older embryos, relatively few cases of two or more embryos in a capsule were found. As these were generally of nearly the same size, it was concluded that if two embryos in one capsule develop unequally the smaller one does not survive. Some capsules have been sectioned which externally appeared to have only one embryo but in reality two were present, one of which might have stopped its growth very shortly after implantation. One case showed two embryos at different stages of development, one at a right angle to the other. Various types of malformation have also been observed in embryos of 8 days gestation as well as in earlier stages.

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EXPLANATION OF PLATES

R and L refer to right and left uterine horns. Numbering of embryos begins with the one next the vagina.

PLATE 1

EXPLANATION OF FIGURES

Ventral views of right and left uterine horns after removal of ventral half of capsules. In some figures only one horn of a given uterus is shown. All embryos are from the Bagg-albino strain. $\times 4$.

Figures 1-5, 8-day embryos. Figure 6, $8\frac{1}{2}$ -day embryo.

1 and 2 Series 8-5.

3 Left horn of 8-8.

4 Left horn of 8-1.

5 Right and left horns of 8-7.

6 Left horn of $8\frac{1}{2}$ -1, with one partially dissected embryo, R-1.

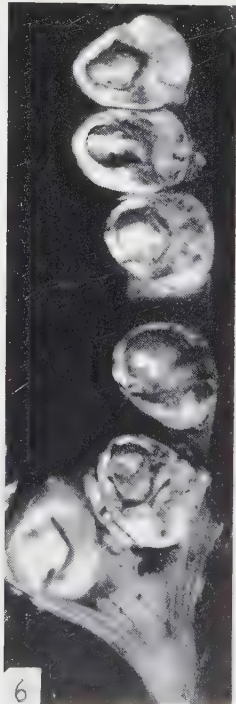
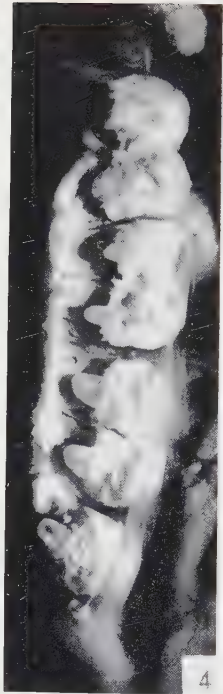
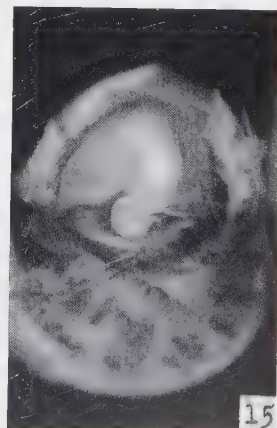
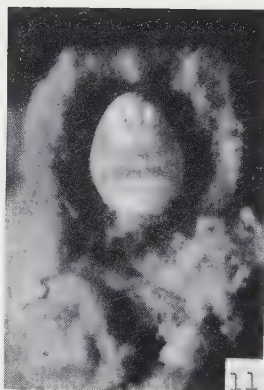
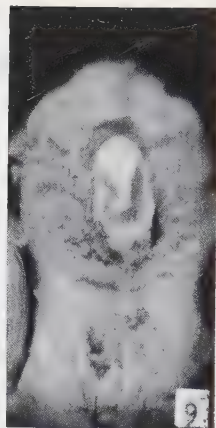


PLATE 2

EXPLANATION OF FIGURES

Individual embryos from litters (with one exception) shown in plate 1. $\times 12$.

7	Series no. 8 8, embryos L-6 and 7 (compare fig. 3).	
8	8-7	L-2 (compare fig. 5).
9	8-8	L-2 (compare fig. 3).
10	8-1	L-2 and 3 (compare fig. 4).
11	8-7	R-6 (compare fig. 5).
12	8-7	L-1 (compare fig. 5).
13	8-2	L-5 and 6 (not shown in plate 1).
14	$8\frac{1}{2}$ -1	L-4 (compare fig. 6).
15	$8\frac{1}{2}$ -1	L-5 (compare fig. 6).



MIRROR IMAGE COMPARISON OF UPPER AND LOWER JAWS IN PRIMITIVE TETRAPODS

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TWO FIGURES

In the higher vertebrate classes upper and lower jaws are not particularly comparable in structure. Work on lower tetrapods, however, leads to a realization that in primitive amphibians and even in many early reptiles upper and lower jaws are essentially mirror images of each other. The outer surface of the lower jaw is comparable, in the number, nature and position of the bony elements present, with the cheek region of the skull; the inner surface of the jaw is closely comparable in its pattern to the primitive tetrapod palate.

This comparison is apparent in the inspection of the early amphibians shown in figures 1 and 2 (many other genera could well have been substituted for those illustrated). In making this comparison the "rostral" elements of the skull—premaxillae and nasals—are disregarded, since it is generally agreed that the primitive condition was one in which the jaws did not extend forward beyond the nares; the rostral region is still in a state of flux in crossopterygians and even in many early amphibians, clearly indicating that forward jaw extension was a late innovation.

On the outer surface of both upper and lower jaws (fig. 1) the main tooth-bearing element A (maxilla or dentary) is a prominent and conspicuous member. In the form figured the maxilla is somewhat smaller than the dentary; in some cases, however, it is larger and extends back, as does the dentary to gain a contact with element E. In both upper and lower jaws we find, adjacent to the maxilla or dentary, and

continuing back to the articular region, a series of four other dermal elements, B to E, including in the upper jaw the lacrimal, jugal, squamosal and quadrato-jugal, and in the lower jaw splenial, post-splenial, angular and surangular. Elements B and C are adjacent to the main tooth-bearing element; D is always large, forms much of the rounded posterior angle of the jaw and is in contact along this margin with element J in both upper and lower jaws. E tends to be relatively small, forms the posterior portion of the dental margin

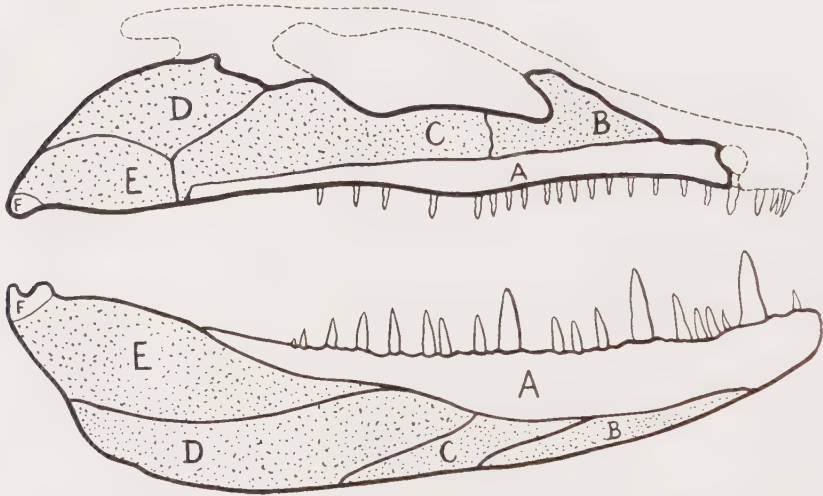


Fig.1 Above, cheek region of the carboniferous labyrinthodont amphibian, *Orthosaurus*; skull outline completed in dashed lines. Below, external view of lower jaw of the same. Both after Watson (1926, figs. 7, 10). In upper jaw: A, maxilla; B, lacrimal; C, jugal; D, squamosal; E, quadrato-jugal; F, quadrate. In lower jaw: A, dentary; B, splenial; C, postsplenial; D, angular; E, surangular; F, articular.

of the jaw, and is tightly braced against the articular element F.

Marked differences are to be found only as regards the "peripheral" margins of elements B and C: This is to be expected because of their relationships to other structures. Below, B and C merely form part of the lower border of the jaw; in the upper jaw the corresponding elements are modified

in connection with articulations with the bones of the skull roof and the presence of the orbit.

This comparable arrangement of external elements might be attributed to pure chance were not the similarities reinforced by a more fundamental mirror image comparison of the inner surfaces of the jaws (fig. 2). In each case the margin consists of a narrow tooth-bearing band formed by element A, behind which element E (and sometimes C in the upper

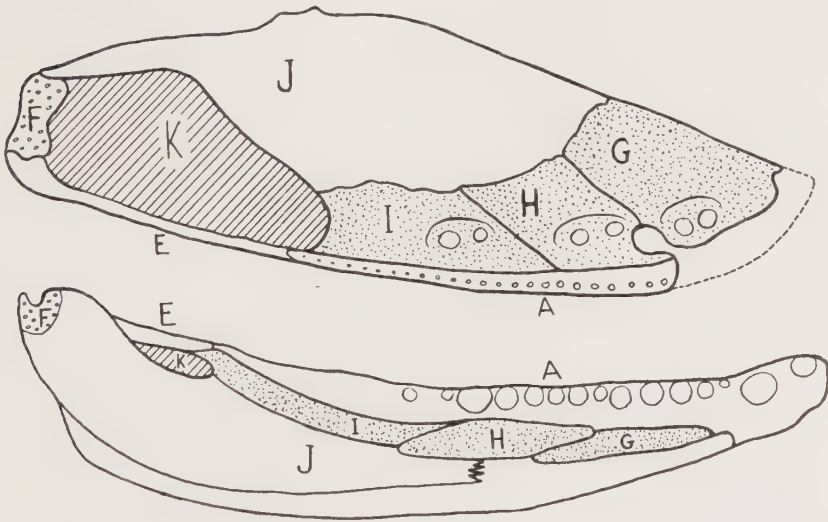


Fig. 2 Above, left side of palate of the carboniferous labyrinthodont *Baphetes*, the premaxilla in dashed outline and the braincase omitted. Below, inner side of the left mandible of the related genus *Orthosaurus*; teeth omitted. Both after Watson (1926, figs. 11, 4A). In palate: A, E, F' as in figure 1; G, prevomer; H, palatine; I, ectopterygoid; J, pterygoid; K, subtemporal fossa. In lower jaw, E, A, F' as in figure 1; G, H, I, three coronoids; J, prearticular; K, meckelian fossa.

jaw) continue back to the cartilage bone F' forming the articular surface—quadrate in the upper jaw, true articular element in the lower. In either case a large fossa (K) is present posteriorly, the subtemporal fossa in the upper jaw, meckelian fossa in the lower. In the lower jaw a dental foramen, carrying blood vessels and nerve, enters posteriorly, typically piercing element J and (in some cases at least) enter-

ing the fossa between E and F. In the upper jaw there is frequently found a quadrate foramen entering the fossa posteriorly between elements E and F. The functions of this foramen are unknown but must surely have been similar, in part at least, to those of the lower jaw foramen.

Internal to element A is a longitudinal series of three bones, G, H, and I, comprising the coronoid series in the lower jaw and in the upper termed prevomer (or vomer?), palatine and ectopterygoid. These three are tooth-bearing in all primitive forms. In both upper and lower jaws they carry a shagreen of tiny "denticles." In the upper jaw each bone also typically bears in primitive amphibians, a large fang and a pit for the replacing tooth. In the lower jaw these are absent in amphibians; but in the crossopterygians, from which (in a broad sense) tetrapods surely arose, similar fangs and adjacent pits are present on the lower jaw series also (cf. Watson, '26, fig. 38). Element G differs somewhat in the two jaws, for in the upper jaw it extends forward into the "sub-rostral" region and meets its fellow of the opposite side.

The innermost element, J, is, in both cases, very large and long; this is the pterygoid in the upper jaw, the prearticular in the lower. The pterygoid commonly bears teeth; the prearticular may do so, but less frequently. Both are intimately associated posteriorly with the respective articular elements, and appear to have arisen in connection with the primary palato-quadrate bar; both form a deep medial boundary for fossa K and are connected with element D; both extend well forward inside of the series G, H, and I. At the margins farthest from the tooth row, however, the upper and lower elements differ in their middle and anterior portions, for the prearticular of the lower jaw is simply connected with the external elements at the lower margin of the jaw, while the pterygoid enters into the articulation of upper jaw with braincase.

Upper and lower jaws are thus seen to be similar to one another both externally and internally in the arrangement

and character of the bony elements present. Demonstration of this mirror image relationship proves didactically useful. We have, however, no adequate explanation for the presence of these structural similarities. Certain points may be interpreted on functional grounds; it is, for example, reasonable to expect in both jaws a single large element forming a base for the marginal tooth row. But no such explanation will account for most of the other common features; proof that they are not of functional importance is given by the fact that later tetrapods tend to modify them greatly. Similarity may be due to inheritance from some simple and primitive ancestral stage, but our knowledge of the piscine ancestors of the tetrapods is very inadequate. Perhaps the presence of similar adult features may be the result of similar conditions and forces operating ontogenetically in both upper and lower jaws; but unfortunately embryos of extinct paleozoic amphibians are not available.

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REGARDING THE EFFECTS OF DESOXY-CORTICOSTERONE AND OF TESTOSTERONE ON THE ADRENAL X ZONE

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ONE PLATE (SEVEN FIGURES)

The characterization of the X zone as a transitory differentiation of the adrenal cortex of mice which is not the equivalent of the permanent cortex is supported both by morphological and physiological considerations (Howard, '27, '37, '39). Thus there are differences in the appearance, arrangement and staining intensities of the cells. The formation of the X zone is a process in the life of the adrenal which ordinarily occurs at a particular time, but which is subject to modification. The X zone is maintained for periods of time which vary according to certain factors, and then undergoes degeneration. If one adrenal is removed when the two adrenals are in a state of beginning degeneration, the degeneration is not halted, and may even be accelerated. The apparently homologous degeneration of the inner part of the cortex in the human during the first few months of life results in a 50% decrease in the weight of the gland (Scammon, '26). However, X zone differentiation can be stimulated at various times in the life cycle by castration. In view of these facts, the spontaneous X zone degeneration has been regarded as more probably representing a qualitative change in the adrenal than merely reflecting a quantitative decrease in the need of the organism for cortical hormone.

It was observed by Deanesly and Parkes ('37) that the hypertrophied X zone of castration can be prevented from

developing, or caused to disappear after it has developed, by injections of various androgens. Furthermore, a certain degree of stimulation of the seminal vesicles in castrated mice has been shown to be associated with the presence of the X zone in the adrenal (Howard, '37, '39). These facts suggest that the X zone may be concerned with a type of sterol metabolism in which androgens are produced.

Evidence purporting to negate this conception of specialization in the function of the X zone has recently been recorded by Gersh and Grollman ('39). These authors reported that injections of cortical hormone caused degeneration of the X zone. Their photographs of adrenal sections are difficult to interpret except as evidence of quite variable material. It has been shown by Ingle, Higgins and Kendall ('38) that the rat cortex atrophies after the administration for 6 to 14 days of 3.3 mg. per day of corticosterone in drinking water. Since the X zone is a differentiation of the cortex which probably differs in the relative amounts of sterols which it secretes, rather than in a complete qualitative peculiarity of hormone products, one would expect that it would share to some extent in any generalized cortical response. However, if it has a greater sensitivity to one class of sterol hormones than to another, this would indicate a degree of specificity in the nature of its cells.

Accordingly I have made a comparative study of the effects of desoxy-corticosterone acetate and testosterone propionate on the X zone.¹ Female mice were chosen since the X zone is normally much better developed and lasts for a longer period of time in females than in males. The mice used were from the H strain which has been bred in my laboratory with a uniform diet for 4 years. The animals were injected daily with varying amounts of the hormones in 0.05 cc. of corn oil, intraperitoneally, and were killed 24 hours after the last injection. The data are summarized in table 1, and

¹The synthetic desoxy-corticosterone acetate and testosterone propionate used in these experiments were supplied by the Ciba Pharmaceutical Corporation through the courtesy of Dr. E. Oppenheimer.

photographs of typical sections are reproduced in plate 1. The glands were fixed in Bouin's, sectioned serially and stained in haematoxylin. It was found that treatment with a tenfold range of concentration of desoxy-corticosterone acetate, up to 0.5 mg. daily for 14 days, did not produce any marked degree of cortical atrophy nor any appreciable inhibition or qualitative change in the appearance of the X zone. (Compare figs. 1 and 3.) Testosterone propionate, on the other hand, after only 6 days' treatment with 0.5 mg. daily, produced degeneration and in some cases almost complete disappearance of the X zone, and after 10 days' treatment

TABLE 1

Contrast in the effects of injections of desoxy-corticosterone acetate (D) and testosterone propionate (T) on the adrenal X zone

TREATMENT	DAILY DOSE	NO. OF MICE	AGE INJECTIONS BEGUN	DURATION OF INJECTIONS	ADRENAL, 1 DAY AFTER INJECTION PERIOD, CONTAINED
	<i>mg.</i>		<i>days</i>	<i>days</i>	
D	0.5	6	23 to 30	14	Well developed X zone
D	0.1	4	24 to 30	14	Well developed X zone
D	0.05	4	24 to 30	14	Well developed X zone
T	0.5	4	24 to 27	10	X zone absent except for traces
T	0.1	4	24 to 27	10	Absent or degenerating X zone
T	0.05	4	24 to 27	10	Absent or degenerating X zone
T	0.5	5	26 to 27	6	Degenerating X zone
Controls	—	6	—	—	Well developed X zone

considerable degeneration was produced with all three concentrations. However virtually complete disappearance of the X zone in all individuals was only produced by 0.5 mg. daily for 10 days. (See fig. 2.) It has been stated by Starkey and Schmidt ('38) that a single intramuscular injection of 0.4 mg. testosterone propionate in oil will cause complete removal of the X zone by the sixth day after the injection. This we did not find to be the case with intraperitoneal injections, as five mice, treated with 0.5 mg. testosterone propionate in one injection, and killed on the sixth day, showed in all cases well developed X zones with no signs of degeneration. Our find-

ings are accordant with the data of Deanesly and Parkes ('37) regarding the effect of androgens on the X zone.

Testosterone thus causes a very rapid disappearance of the X zone, which constituted in these mice about one-third of the cortex. In the 6 and 10 day experimental periods these animals gained about 30 and 40%, respectively, of their original body weights. This rate of change in tissue quantity suggests that the cells of the X zone may actually be removed during these short periods. However, it may be that under some conditions the X zone cells are, at least in part, simply transformed by testosterone injections to cells of the typical appearance of those of the permanent cortex. This possibility was suggested by the observation that in two animals which had received respectively 0.1 mg. and 0.05 mg. testosterone propionate for 14 days, the X zone was clearly recognizable as a large area in which the cells were unusually large, with, in one animal, a more vacuolated less intensely staining cytoplasm in some cells, strongly suggestive of a process of transformation to zona fasciculata cells. On the other hand, in one animal, which had received 0.5 mg. testosterone daily for 6 days, there was a fairly large degenerating X zone in which the cells were very small and apparently compressed. In all other animals the zone had either entirely disappeared or was represented by a narrow region of compressed degenerating cells.

The finding that desoxy-corticosterone acetate, injected for a somewhat longer period than testosterone propionate, did not cause appreciable alteration in the X zone, within the concentration range employed, contradicts the observations of Gersh and Grollman ('39). These authors administered desoxy-corticosterone acetate, 0.5 mg. daily intraperitoneally in oil to mice, which, as indicated in their table, were considerably younger than those used in the present experiments. In such young mice the X zone is at best very small, and usually, in my experience, histologically ill-defined in comparison with its fully developed state. In the present experiments, working with mice in which the X zone was well

defined at the beginning of the injection period, but still within the period in which in females the X zone is normally undergoing rapid enlargement relative to the permanent cortex, we have been able to show that within a tenfold concentration range in which testosterone propionate causes X zone disappearance or degeneration, desoxy-corticosterone has no appreciable inhibiting effect.

Gersh and Grollman also used some older animals treated with a charcoal adsorbate of a cortical extract. In view of the isolation of an androgen from cortical extract (Reichstein, '36) there is a possibility of androgens being present in cortical extracts. Although androgens are only very slightly active on oral administration (Womack and Koch, '30), the ease with which androgens are absorbed from the skin (Moore, Lamar and Beck, '38) suggests the possibility that mice treated with a charcoal adsorbate of a cortical extract in their food might have absorbed cutaneously and orally sufficient androgen to cause some X zone degeneration. These possibilities make it preferable to work with pure substances in this type of experiment. Other considerations which should also be borne in mind in evaluating this work are that in some strains of mice the X zone is not well developed (Howard, '38) and in my experience very variable manifestations of the X zone have occurred under some conditions.

In evaluating the physiological significance of the quantities of desoxy-corticosterone acetate used in the present experiments, they may be compared with the dose required to give adequate maintenance in adrenalectomized animals. Ingle ('40) reports that desoxy-corticosterone acetate is the most active of the known steroid compounds in its life maintaining capacity, but inferior in promoting ability to do muscular work. The growth maintenance requirements of mice, under the conditions of my experiments, were as follows. Seven adrenalectomized mice (15 gm.) were partially maintained but with subnormal growth on 0.02 mg. per day, given in daily injections in oil intraperitoneally. Four adrenalectomized mice in which subcutaneous pellets of the hormone had been

implanted, grew at an entirely normal rate and were maintained for 3 weeks in excellent general condition. Untreated adrenalectomized controls did not gain in weight and lived an average of 7 days. Hormone was lost from the pellets at rates of 0.1 to 0.2 mg. per day per mouse. Further details regarding substitution of the adrenals with desoxy-corticosterone acetate will be published elsewhere.

The effect of pellets of desoxy-corticosterone acetate on the developing X zone was studied in infantile mice. Four mice were castrated at 4 days of age, and two given subcutaneous pellets at the time of operation. All were killed at 19 days of age. Sections from these glands are illustrated in figures 4, 5, 6, and 7. In castrates the X zone is well developed at this age. The desoxy-corticosterone treatment was sufficient to cause some cortical inhibition, as evidenced by the smaller size of cells in the zona fasciculata. Thus counts of nuclei showed a 40% increase in the number of cells per unit cross-section area in the zona fasciculata of the treated mice as compared to the untreated. Nevertheless the X zone was clearly differentiated, of normal size relative to the permanent cortex, and did not appear to have undergone any greater inhibition than had the cortex as a whole.

The entire significance of the X zone cannot be evaluated at present. As a differentiation of the permanent cortex it may be expected to respond to certain influences in a manner paralleling the response of the permanent cortex. However, the point made by the evidence of this communication is that the X zone promptly disappears following treatment with an androgen, but is not appreciably affected by similar concentrations of a sterol which manifests the growth maintaining activity of the adrenal cortex. Other compounds with adrenal cortex replacing activity should be tested with regard to their effect on the X zone, when such substances become available in a pure state.

Estrogens and progesterone likewise do not cause X zone disappearance (Howard and Gengradom, '40). It appears, then, that early X zone atrophy is a reaction somewhat specific to androgen.

This should not lead one to infer that some degree of a similar response to androgen does not become manifest in the cortex as a whole. For example, the cortex has been found to be larger in females than in males (mice, Carlson, Gustafsson and Moller, '37; rats, Jackson, '19, Donaldson, '19, Andersen and Kennedy, '32; dogs, Baker, '37; and man, Swinyard, '40), and enlarged in male rats after prepuberal castration (Andersen and Kennedy, '33) although a definitive X zone does not differentiate in castrated rats (Howard, '38). These size differences may be a result of the factors which affect the X zone. One probably should not regard the X zone as a tissue entirely different from the permanent cortex, but as a tissue in which certain properties of the cortex are particularly well developed. Hence a study of the physiology of the X zone should be of value in an analysis of cortical functions.

SUMMARY

1. The adrenal X zone promptly degenerates following treatment with testosterone propionate.
2. The X zone is not appreciably affected by equal concentrations of desoxy-corticosterone acetate.
3. This difference in X zone response accords with other considerations which indicate that the X zone is probably not functionally equivalent to the permanent cortex and may be more concerned with andromimetic activity.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

1 Adrenal of uninjected control female showing: above, permanent cortex; middle, dark staining X zone; below, medulla. The cortico-medullary boundary is indicated by ink marks.

2 Adrenal of female injected for 10 days with testosterone propionate (0.5 mg. daily). The X zone has disappeared, and there remain the structures typical of the permanent cortex (above) and the medulla (below) with a clear-cut line of demarcation.

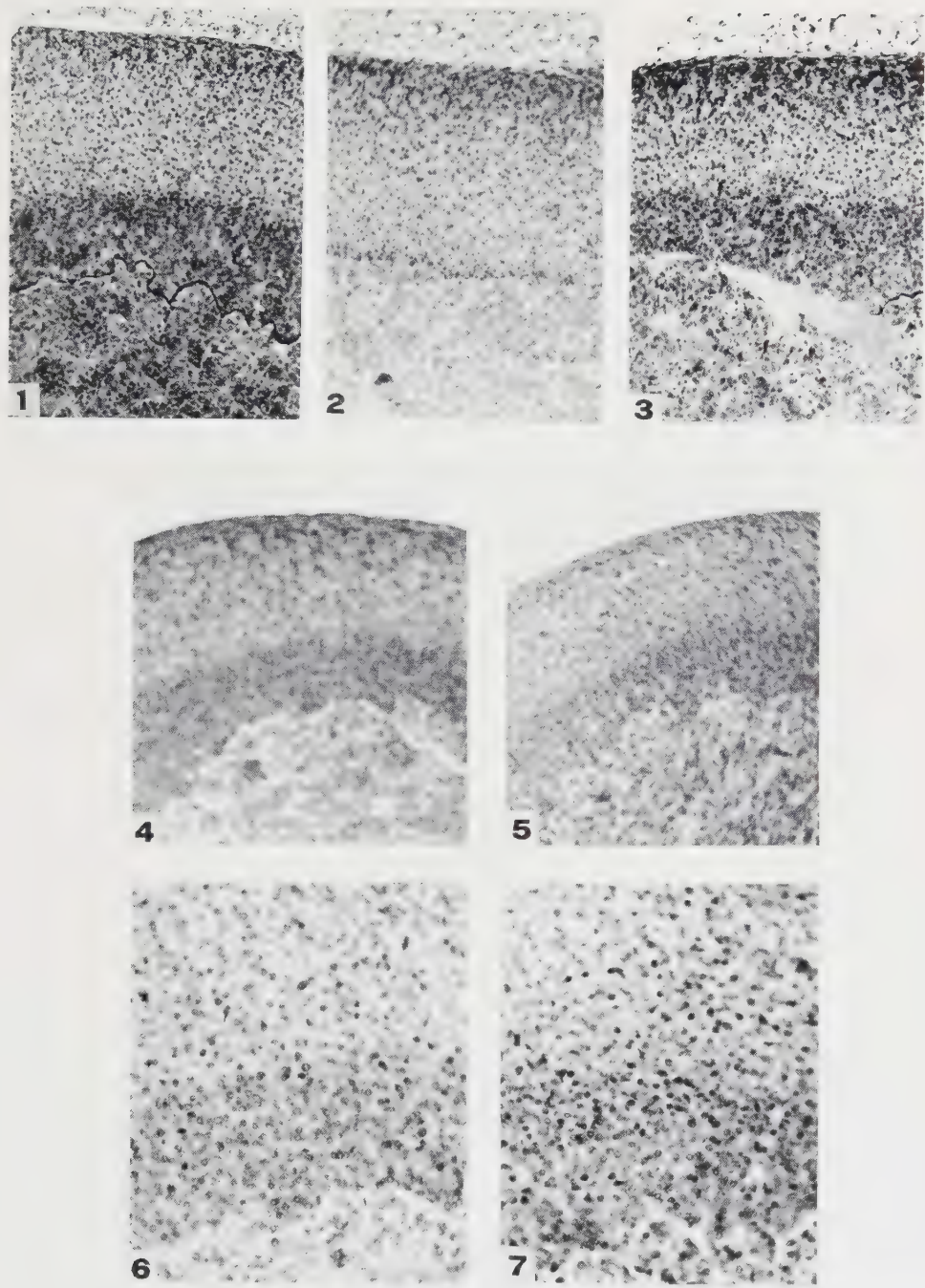
3 Adrenal of female injected for 14 days with desoxy-corticosterone acetate (0.5 mg. daily), showing: above, permanent cortex; middle, dark staining X zone; below, medulla. The large vein follows the lower edge of the X zone across most of the section. The cortex is somewhat narrower, but the X zone has not been appreciably suppressed, in contrast to the effect of testosterone shown in figure 2.

4 Adrenal of male castrated at 4 days of age and killed after 15 days. Note presence of moderately sized well differentiated X zone.

5 Adrenal of male castrated and implanted with a pellet of desoxy-corticosterone acetate at 4 days of age, and killed after 15 days. Note differentiated X zone similar in size and general appearance to that shown in figure 4.

6 Higher magnification of adrenal in figure 4, showing zona fasciculata (above), darker staining X zone (middle), and a little of lighter staining medulla (below).

7 Higher magnification of adrenal in figure 5. Zones as in figure 6. The cells of the zona fasciculata are somewhat smaller after the desoxy-corticosterone treatment.



A COMPLETE UNEQUAL TWIN IN A TERATOMATOUS TUMOR OF A BELGIAN HARE

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Incidence of teratomatous tumor in the rabbit is not uncommon as evidenced by the large number of cases encountered annually in the dissecting laboratory. The majority of such tumors are in the nature of dermoid cysts occurring in numerous locations in the bodies of the animals affected. Such cysts may contain cheese-like masses of amorphous substances or, less often, disorderly collections of tissues and organs. In instances, a more or less poorly developed fetus may occupy the cyst. Such an embryoma may be referred to as a fetus in fetu. The larger individual is referred to as the "autosite," the smaller is considered a parasite. Often, the parasite is not entirely included within the body of the autosite, but the two are conjoined over a large or small area, and important organs may be possessed in common. Although there is a union between the individuals, the case clearly presents itself as involving identical twins. While the dermoid cyst appears, on cursory examination, to bear no relationship to the affected individual comparable to that of twin to twin, the pathological conditions are homologous. Consideration of this problem lies in the sphere of pathological embryology.

The purpose of this paper is to record the occurrence of an anatomically complete and evidently normal rabbit fetus completely encysted in the abdominal region of an adult female rabbit. The rabbit was procured at a biological supply house, embalmed and injected for dissection purposes, and many observations which might have proved of incalculable value were rendered impossible. A second fetus, normal development of which had been arrested so early that it was recognized with difficulty, was present in the same cyst, appearing as an amor-

phic mass much smaller in size, molded to the contour of the normal fetus at the base of the thoracic vertebrae of the latter, but anatomically separate.

The cyst lay ventrally in the mid-line and to the right of the mid-line in the abdominal region, 11 cm. caudal to the xiphoid process, and 9 cm. anterior to the insertion of the inguinal ligament on the symphysis pubis. Shaped like a fore-shortened dumbbell, it occupied a position perpendicular to the linea alba, with one pole of the dumbbell occupying the abdominal cavity, the opposite lying in the ventro-lateral body musculature between the cutaneous maximus and the external oblique muscles. Occupying the coelomic pole were the head and thorax of the fetus, whereas the pelvic girdle and posterior appendages occupied that portion of the cystic cavity within the body wall. The fetus was greatly constricted at a point immediately posterior to the inferior thoracic aperture, to occupy that portion of the cyst passing perpendicularly through the linea alba. When the cyst was removed, there remained in the mid-ventral body wall of the autosite a foramen 1.4 cm. in the longitudinal axis of the autosite, and 0.3 cm. wide, with a smooth, glistening border, and passing through the linea alba and underlying aponeuroses.

The crown-rump length of the fetus was 6.2 cm.; that of the arrested, amorphous fetus was 1.5 cm.

The cyst was lined with thin, tough, connective tissue. That portion of the lining containing the cephalic region of the fetus, and projecting into the abdominal cavity, adhered to the head and trunk of the fetus; but the lining of the opposite pole of the cyst was inextricably fused with the fascia of the internal surface of the cutaneous maximus muscle in the region of the anastomosis of the external thoracic and the superficial epigastric arteries. In this fused membrane was located a moderately rich plexus of anastomosing vessels, and through it blood was supplied to the fetus. The superficial epigastric artery was well injected, but the external thoracic artery was poorly injected, indicating that the former channel may have been the principal source of blood. The external thoracic vein ante-

rior to the site of the cyst was somewhat larger than normal. In addition to the connective tissue of the cyst, the fetus was immediately covered by a second thin, transparent membrane.

The condition herein reported was first diagnosed as an extra-uterine pregnancy. A considerable portion of the body of the fetus occupied the coelomic cavity, where the cystic envelop and underlying membrane adhered to the fetus after the fashion of an amnion devoid of fluid. There was, indeed, no evidence to indicate that the inner membrane was not an amnion. An umbilical cord was present in the inter-muscular pole, at which point it was probably continuous with the vascular wall of the cyst. However, in identical twins the umbilical cords are individual, and the same is usually true of the amnions. No reasonable explanation seemed to account for the position of the remainder of the fetal body within, and perpendicular to the long axis of, the body wall musculature of the autosite. It is true that the penetrating power of the embryonic decidual cells as yet has not been fully determined. A rupture in the linea alba, existent at the time of implantation, would possibly account for the inter-muscular implantation of a fetus. If such a trauma had been responsible for the position of the fetus under consideration, it would seem that the developing fetus, situated as it was between two muscular layers would, as growth proceeded, have assumed a position not opposed to the line of least resistance, becoming embedded parallel to, and between, the musculature, rather than wedged perpendicular to the musculature and to the linea alba, as was the case. The actual site of attachment of the fetal membranes to the fascia of the muscle was several centimeters removed from the aperture in the linea alba. Unless the rupture be assumed to have been extensive, this circumstance would appear to have made it necessary for the embryo to have grown toward the trauma in the linea alba; in further growth to have wedged its way through the trauma; and finally to have undergone further differentiation in this perpendicular position—a series of circumstances not inconceivable. Other hypotheses may be advanced to explain the present case as an extra-

uterine pregnancy. The teratological explanation, first suggested by Dr. E. E. Reinke, of the Department of Biology, Vanderbilt University, presents itself as logical. Belief that the fetus and the autosite were twins was strengthened by the observation that the parasite was identical with the autosite in sex, and color of hair. No distinguishing pattern was present on either. It may be stated as a matter of record that the mammary glands of the autosite were in a resting condition.

To existing knowledge of the etiology of twinning, and of teratogenesis, Stockard ('21), Newman ('23), and Streeter ('24) have made valuable contributions. In the opinion of Maccallum ('24) evidence greatly favors the theory of isolation of blastomeres as the origin of teratomatous tumors. A totipotent blastomere, isolated from the developing embryo at an early period, may become completely surrounded by the developing autosite so as finally to be included in the body of the latter. Development of this independent blastomere at a later date would then result in an unequal included twin, or conjoined twins.

According to Stockard, "when the two components of a double specimen are unequal in size, the larger component is almost always normal in structure, and the smaller component is always deformed." The details herein set forth are designed to serve as a record of a complete and apparently normal fetus in fetu.

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THE PNEUMATIZATION OF THE HUMERUS IN THE COMMON FOWL AND THE ASSOCIATED ACTIVITY OF THEELIN

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TWO PLATES (SIX FIGURES)

In a study of the pneumatization of the bones of the chick head it was found that the air sac, in this case the tympanic cavity, always expands into loose mesenchymal tissue. In the membrane bones of the cranium the wall of the enlarging cavity meets the scattered developing bone spicules and bulges around them in a series of deep bays, which spread further between the trabeculae and anastomose with each other to form a single cavity, bounded by the inner and outer cortical tables and crossed by the trabeculae of the diploë, many of which are later resorbed. The air sac simply expands in the mesenchyma as the bone is being formed. In the case of the bones of the base of the skull and of the face, bones which are preceded by cartilage images, the walls of the tympanic cavity, either directly or by means of tubular processes growing out from the cavity, are first applied to the surface of the cartilage, then bulge into the primary marrow spaces of the future bone as the underlying cartilage is dissolved, and finally are buried beneath the perichondrial bone of the cortex as the latter grows over it from the sides. The cortex is formed around the air sac, as the latter expands in the mesenchyma of the primary marrow.

It has long been known that in many birds some of the long bones of the limbs are pneumatized by the ingrowth of tubular processes from certain of the voluminous air sacs connected with the lungs, and that the extent of pneumatization varies widely in the different classes of birds. As a rule,

to which there are noteworthy exceptions, the better fliers show the greatest aeration: in the common fowl the humerus is the only limb bone affected. The development of the air sacs has been studied by Locy and Larsell ('16), who, however, carry the growth to the axilla only. The pneumatization of the bones was described by Strasser and by Wildermuth, both writing in 1877, and by Blumstein-Judina, a pupil of the former, in 1905. Since then, according to Nauck ('38), no further investigation of the subject has been undertaken. Blumstein-Judina confined her description to the extension of the air sac within the marrow cavity after its initial entrance, and left the early stages to be re-described by Strasser in a further paper, which, however, apparently never appeared.

As regards the actual entrance of the air sac into the bone the two earlier authors differed in opinion. Strasser maintained that the cortex in question was weakened locally by its position between the muscles and the joint and by being therefore submitted to constant movement during osteogenesis, and that the entrance of the sac was due to simple pressure on this weakened area of bone. Wildermuth found that the fibrous coat of the sac combined with the periosteum of the bone to form jagged, knobby processes which pressed against and ate into the cortex, causing a corroded appearance. "The picture reminds one exactly of the penetration of a pathological neoplasm." Of these two conceptions the latter is more nearly correct, as the following observations will indicate.

In the avian humerus the site of the minute opening in the cortex through which the air sac passes, the "porus pneumaticus," is on the medial side of the diaphysis just below the proximal epiphyseal line. Since the bone is growing in length in the period after hatching when the air sac effects its entrance, this position is constantly shifting. It is bounded below by the edge of the belt of cortical bone which has already surrounded the shaft of the cartilage image and above by the cartilage of the subepiphyseal region. It is the site of the most active bone development and hence of the most recently

deposited bone matrix. Small vessels pierce the perichondrium at several points around the circumference of the bone at this level, the largest of them on the medial side where they join the axillary vessels. In the adult bird similar groups of vessels, mostly veins, persist in many of the long bones and form an accessory drainage for the main nutrient artery, as shown by the careful injections of Doan ('22). The layers of the cortex, as the bone elongates, take the form of perforated parallel sheets, connected at intervals by short radial bars, and slanting outward toward the epiphysis, a disposition associated with the fact that the cartilaginous epiphysis is broader than the shaft of the bone, thus imparting an elongated funnel shape to the newly formed superposed periosteal lamellae. The veins follow the slanting diagonal direction of the primary marrow spaces between the cortical sheets; they open into the main marrow cavity nearer the middle of the shaft, but may have side branches from the marrow along the way.

The expanding axillary air sac, composed of simple cuboidal or flat epithelium resting on a delicate connective tissue membrane, burrows through the areolar tissue between the muscles of the axillary region, advancing by a broad margin which wraps itself around the solid structures encountered, and thus becomes divided into irregular lobes at its growing edge. On approaching the humerus it branches around the teres major muscle near its attachment to the bone, some distance below the epiphyseal line. The lower bay of the air sac meets the periosteum, the connective tissue of the sac fuses with it, and the sac flattens against the bone, but does not enter it. Instead, the sac wall becomes wrinkled, the epithelium thickens, and new side pockets burrow between the muscles at some distance from the bone, seeking new regions in which the sac may expand. The apposition of the air sac to the bone cortex is not in itself sufficient to ensure entrance into the bone.

The upper bay of the air sac, above the teres major, meets the bone at the level of the exit of one of the veins, in the

region of the newly formed cortex. At a period ranging from 12 to 20 days after hatching, in the chick (the time is very variable), the periosteum and the cortical primary marrow in this region are undergoing specific changes. The osteoblasts lying against the bone spicules disappear, osteoclasts are seen in increasing numbers, the trabeculae are gradually resorbed and the inner layer of the periosteum is replaced by a delicate tissue closely resembling mesenchyma. These changes are at first limited to the area immediately surrounding the vein exit (fig. 1), but they extend inward chiefly along the vein, with the result that the latter soon lies in a constantly enlarging tubular compartment, surrounded by bone trabeculae, filled by mesenchyma, and slanting from the outside surface of the bone above to the marrow cavity nearer the middle of the shaft. The mesenchyma becomes looser in texture and irregular spaces or cysts develop in it. Into this loose tissue, along the vein, the air sac finally grows in the form of a long tube (fig. 2). If some more solid structure has remained in its way, such as a strand of denser periosteum or an arteriole or a lateral branch of the vein, the sac may divide around the obstruction and be branched or double. This would account for the multiple *pori pneumatici*, occasionally found, and for the cribriform area of entrance as described by Wildermuth.

The actual entrance of the air sac into the main marrow cavity is effected at first at the internal opening of the vein. The histological changes marked by the reduction of osteoblasts and their replacement by mesenchymal tissue extend by progressive expansion from the inner wall of the cortex onto and along the marrow trabeculae, forming a sheath of mesenchymal tissue around the latter (figs. 3 and 4). The typical marrow is thereby reduced to central masses, separated from the bone trabeculae by this mesenchyma. The air sac, always following the mesenchyma, sends flattened branches along these extensions and as the branches grow larger they tend to encircle the contents of the intertrabecular spaces and to enclose isolated islands of normal unaltered

marrow. Some of the marrow degenerates when surrounded merely by mesenchymal tissue, some after being encircled by the flattened branches of the air sac (fig. 5). In the latter case the marrow usually retains its own blood supply, and the arrangement may be compared, as to the relations of the component parts, to that of the renal glomerulus with its vascular pole, central mass, and surrounding double-walled capsular space. Finally the bone trabeculae which traverse the marrow cavity are for the most part resorbed, as well as the inner layers of the cortex. The various branches of the sac fuse and anastomose, and the resulting large single air sac is seen to enter the bone directly from the external pore and to occupy most of the bone cavity, relegating the marrow to the two ends of the shaft.

The relation between the air sac and the enclosed marrow is difficult to determine, as noted by Blumstein-Judina, because of the extreme delicacy of the epithelium, but the study of the reflections of the sac wall reveals the true condition. The relation of the sac to the enclosing cortex is variable. In some regions the connective tissue layer of the sac forms the actual endosteum of the cortex, in others the epithelium is separated from the bone by large spaces, considered by Wildermuth as "lymphatic." The conditions probably represent, in the one case, the complete loss of the mesenchymal sheet through which the air sac makes its growth, and, in the other, the retention of this sheet in cystic form.

The microscopic picture seen in the expanding zone of the active bone changes beyond the limit of the entering air sac reminds one inevitably of similar pictures seen in the mammalian bones which have been altered by the disease called *osteitis fibrosa cystica*. The presence of a mesenchymal, cystic connective tissue surrounding the bone trabeculae and impinging from all sides on islands of degenerating bone marrow, the reduction in the number of osteoblasts and the increase of osteoclasts are all part of the classical descriptions of this pathological entity. The disease, usually accompanied by an increase of calcium in the blood, has been

proved by many authors to be due to the increased activity of the parathyroid glands, and in many mammals can be produced at will by the administration of the gland principle, parathormone (Bauer, Aub and Albright, '29; Bodansky, Blair and Jaffé, '30). The connection of the disease with the pneumatization of the bones in the bird was quite unsuspected.

Most birds are resistant to parathormone treatment, as far as the bone changes are concerned, and reports as to the blood calcium are conflicting. Collip ('31) and Macowan ('32) found parathormone to have no effect on the blood calcium of non-laying hens, while Riddle and Dotti ('34) report that it is effective in pigeons, which "are therefore to be added to the few species now known to be suitable for use in studies" of the parathyroids. The parathyroid glands are not recognized as being unduly large in birds after hatching. Apparently these glands in themselves cannot be responsible for the condition found normally in the chick.

Developmentally, the most striking difference between mammals and birds is the presence in the latter case of a large amount of yolk material. Part of this is used as food for the embryo; the residue, varying in amount in different species, is taken into the intestinal tract when the yolk sac is retracted within the abdominal walls just before hatching. It seemed probable that this large amount of material might in some way be responsible for the bone changes leading to pneumatization. What might be called the direct method of testing this theory, by removal of the yolk sac from the shell before it was retracted, did not seem appropriate in this case, because any change from normal which might be obtained could be perhaps interpreted as merely the result of malnutrition due to the loss of so much normal food. It seemed wiser to seek some active principle in the yolk which might be the responsible agent.

Several facts gleaned from the literature pointed to some female hormone as this agent. Theelin is found in quantity in the yolks of fertile eggs, but not in infertile ones (Riboulleau, '38). Its results are seen in male birds in the ex-

traordinarily small testes normally present at hatching (Riddle, '29), and in the female type of plumage assumed by male chicks of several species for some weeks after hatching (Greenwood and Blythe, '38). As a corollary, the latter authors were able to change the typically male plumage assumed by a previously ovariectomized female back to the female type by injections of egg yolk. Reports as to the effect of estrogens on the blood calcium level are conflicting. Marlow and Koch ('37), pointing to the great normal fluctuations in this level in mammals and fowls, find no significant changes due to estrogen administration, while Altmann and Hutt ('38) maintain that injections of estrogens or of yolk do increase the blood calcium by affecting the parathyroids, either directly or indirectly, and that "their activation results in the higher level of serum calcium characteristic of laying fowls." Riddle et al. ('38) also report that the blood calcium is raised in mammals by sufficiently high dosage of female sex hormones and moreover that this action is strictly dependent on the presence of the parathyroid glands.

The bones also show changes: "in female pigeons there is a cyclic ossification of the red marrow of the long bones of the leg . . . coincident with the maturation of the ovarian follicle" (Keyes and Potter, '34). In mice definite specific bone changes, including decalcification and resorption of the medial portion of the pubic bones, occur in normal pregnancy, and can be artificially induced in both males and females by injections of estrogens. On the contrary, continued treatment with estrogens leads to an accompanying thickening of the other bones of the body, "a general hypercalcification, with loss of marrow," which can be prevented by the administration of testosterone (Gardner and Pfeiffer, '38 a and '38 b). A syndrome including osteitis fibrosa and precocious puberty in females is noted by Albright et al. ('37). Finally a direct connection between the female sex hormone and osteitis fibrosa cystica is suggested by Parhon and Tomorug ('35) in reporting a case of this disease "of parathyroid origin" notably improved by the surgical removal of a cystic ovary. "One

must keep in mind that the parathyroid glands are not the only glands that play a role in calcium changes. The ovaries certainly have their share”

Both in the pigeon bones at the maturation of the ovarian follicle and in the mouse bones after the administration of estrogens the changes reported are not those belonging to osteitis fibrosa but changes due to bone increase; new marrow trabeculae were formed in the one case and in the other the marrow cavities were almost completely filled with bone, very hard and brittle. However, Selye ('32) has shown that the two conditions are actually different manifestations of the same disease. According to his experiments on rats, parathyroid overdosage leads first to bone resorption, osteitis fibrosa, and then, after a period of continued treatment, the same hormone “surprisingly” causes the contrary effect, namely, increased osteoblastic activity and bone apposition, or “marble bone disease.” He explains this phenomenon as the result of “parathyroid hormone immunity” or “tolerance” as he prefers to call it. Burrows ('38) confirms this sequence. With very small dosage of the hormone Selye was able to produce the latter stage directly without previously causing osteitis fibrosa.

Estrogens, then, can cause bone changes identical with “marble bone disease,” and these changes can be prevented by the androgen, testosterone. My first experiments were designed to prove that the air sac gained admission to the interior of the humerus of the fowl only when a mesenchymal pathway had been provided by the action of the estrogens in the yolk to produce the condition of osteitis fibrosa; the attempt was made to block this pathway by the administration of counteracting androgens. Chicks 8 days old were injected with varying amounts of perandren (Ciba) up to a total of 6.5 mlg. in ten daily doses. This total may have been insufficient; the amount of estrogens in the retracted yolk and the rate of its utilization are as yet unknown, and the quantity of the androgens required to counteract their effect must be a matter of pure surmise. Also the fowl, as noted by Riddle

and Dotti, is among those birds resistant to parathyroid therapy. Whatever the cause the results of the experiments were entirely negative. The chicks, both male and female, grew precocious combs and showed the other usual signs of the activity of the surplus androgens, but no influence on the bones could be detected. Pneumatization proceeded normally and at ages well within the normal range.

A second set of experiments sought to prove that estrogens could cause osteitis fibrosa cystica and thus offer the required pathway through the bone. Gardner and Pfeiffer had produced in mice a condition which is probably the final stage of the disease, but reported nothing of the fibrous stage. The solid condition of the bone was noticed in "the femurs of the animals which had received 250 I.U. or more of hydroxy-estrin benzoate or 0.1 mg. of equilin benzoate weekly for 56 or more days." My experiments, some of which were started before I was aware of these authors' results, stressed the earlier stages. Three litters of white rats 30 to 32 days old were given hypodermically injections of theelin in sesame oil (Parke, Davis & Co.). Group I received 4500 I.U. in 2 weeks; group II, 4000 I.U. weekly for 4 weeks (total 16,000 units); group III, 12,000 I.U. weekly for 2 weeks only (total 24,000 units) followed by a period without treatment. The diet was normal. The animals were sacrificed at various ages and the humerus in all cases, the knee-joint and the adjacent bones in many cases were fixed, decalcified and sectioned. Group I gave no significant changes. Group II showed increased bone deposition at 27 and at 34 days after the beginning of the treatment, noticeable in a thickening of the cortex and marrow trabeculae; in this series the fibrous stage was not detected in younger animals. Group III at the twenty-seventh day showed small areas of osteitis fibrosa.

A comparison of figure 6, from the humerus of a rat of group III, with figures 3, 4 and 5 from the humeri of normal chicks indicates that the same, or at least very similar, agents have been at work. The wide canal running diagonally through

the cortex, through which the vein passes, is filled in both instances with a delicate cystic tissue of mesenchymal type which spreads to the marrow trabeculae; osteoblasts are absent or inconspicuous, and osteoclasts are present on the surface of the bone. The air sac is missing in the mammal, but the pathway of easily penetrable material is provided, even in its absence. There can be little doubt that the causative agent is the same in both mammal and bird, and that the avian air sac obtains entrance into the bone because of the timely action of the estrogen contained in the yolk in producing the local disease known as osteitis fibrosa cystica.

SUMMARY

The humerus of the chick is invaded by an extension of the cervical air sac, itself an outgrowth of the lung. The sac reaches the bone only after the cortex is well formed. About 2 weeks after hatching the cortex at several points below the epiphyseal line undergoes changes similar to those characterizing the disease osteitis fibrosa in mammals, with dissolution of trabeculae and invasion by mesenchymal tissue. The invasion spreads along one of the veins to the marrow cavity and extends to the marrow trabeculae. Through this mesenchymal pathway the air sac grows as a tubular process, branching along the trabeculae, destroying the marrow, and finally by anastomosis of the branches occupying the entire cavity.

Osteitis fibrosa is well known to be caused by hyperactivity of the parathyroid glands. A later stage of the disease, with deposition instead of dissolution of bone, has been seen to follow prolonged treatment with estrogens. The present experiments show that the earlier stage may also be produced by estrogens. It is suggested that the estrogens taken into the chick by the retraction of the yolk sac before hatching act either directly or indirectly on the parathyroid glands to produce a temporary condition of osteitis fibrosa, thus affording a mesenchymal pathway for the air sac with resulting pneumatization of the bones.

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PLATE 1

EXPLANATION OF FIGURES

1 Transverse section of humerus, just below proximal epiphyseal line, normal chick, 15 days. Mesenchymal tissue surrounding paired veins as they pierce periosteum and advancing into marrow spaces. Destruction of trabeculae, presence of osteoclasts and cysts. Air sac approaching from the left. $\times 75$.

2 Longitudinal section of upper half of left humerus, normal chick, 16 days. Broad channel through cortex containing mesenchyma surrounding vein (cut twice, with injection mass) and tubular process of air sac (seen in section between two sections of vein). $\times 9$.

3 Lower portion of figure 2. Detail showing cystic mesenchyma extending to marrow trabeculae. Air sac above, vein below. $\times 20$.

4 Similar detail of humerus of chick of 22 days. The expanded air sac has penetrated to the medulla, preceded by fibrosis along the trabeculae. The vein shows as a compressed slit at the left. $\times 20$.

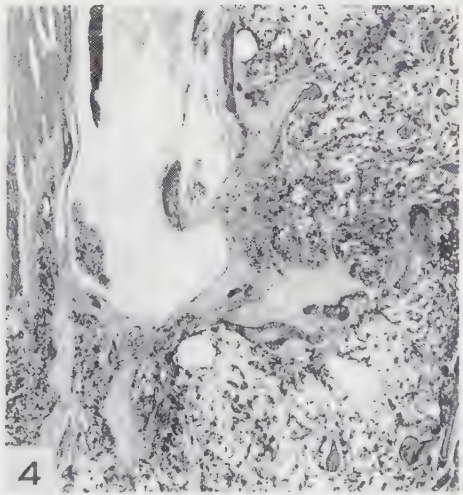
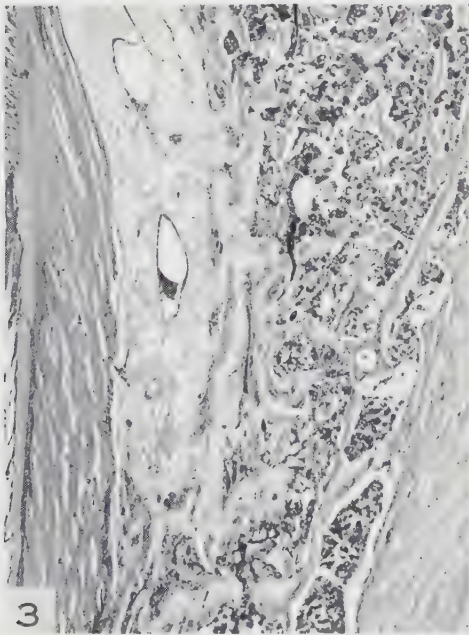
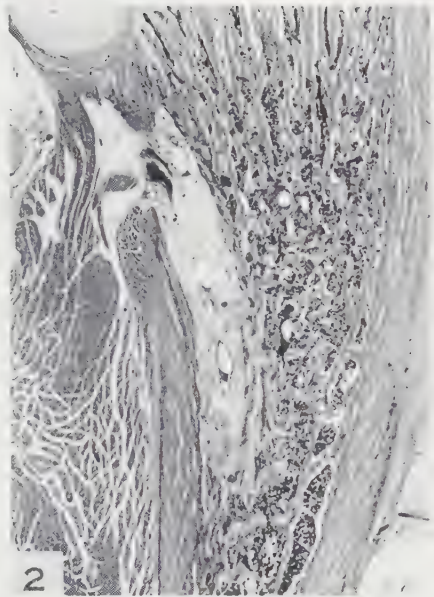
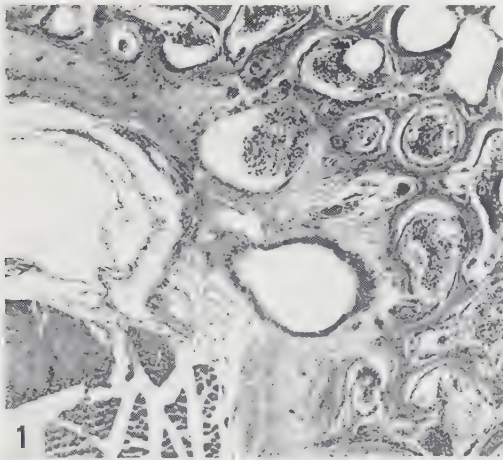
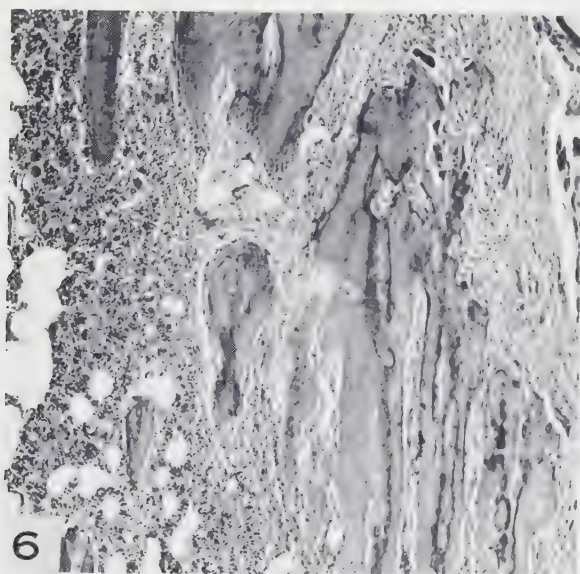
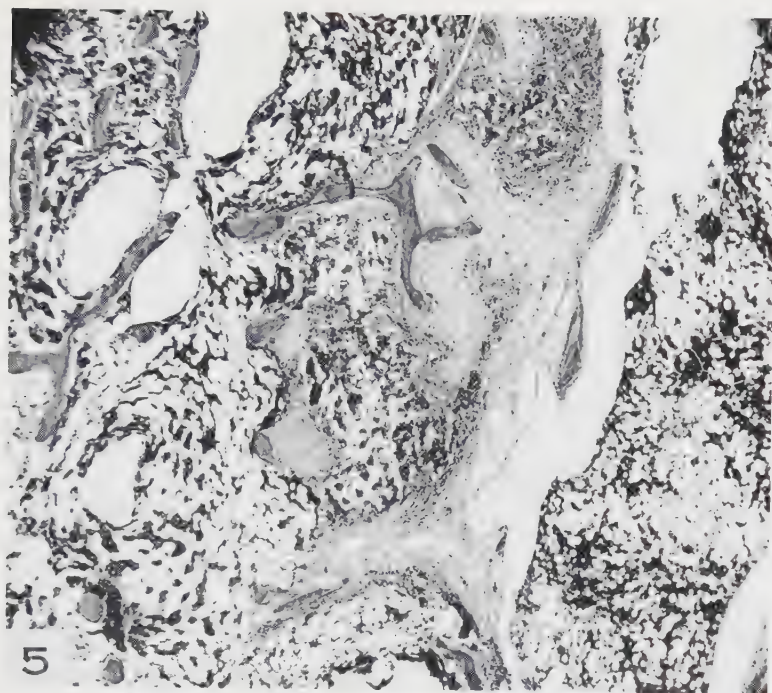


PLATE 2

EXPLANATION OF FIGURES

5 Similar detail of humerus of chick of 18 days. Air sac surrounds a mass of normal marrow and branches into cystic mesenchymal tissue extending further along trabeculae. Marrow encircled by fibrosis sometimes degenerates. $\times 40$.

6 Longitudinal section of humerus of rat treated with 24,000 I.U. of theelin in 27 days. Compare with figures 2 and 3. Similar channel of mesenchyma through the cortex, surrounding a vein and showing cysts, and extension of fibrosis along trabeculae with some loss of osteoblasts. Osteoclasts present at surface of cortex. $\times 75$.



COMPOSITION OF THE RECTUS SHEATH¹

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TEN FIGURES

Descriptions of the rectus sheath contained in our textbooks of anatomy are singularly alike; they are stereotyped and oversimplified; when these accounts recognize structural variation at all it is only in connection with the position of the linea semicircularis. It is regularly stated that in the area superior to the level of the linea semicircularis the internal oblique aponeurosis divides into two lamellae, one blending with the external oblique aponeurosis as it passes in front of the rectus muscle, the other fusing with the aponeurosis of the transversus as it passes behind the muscle; it is further stated that in the territory situated inferior to the level of the linea semicircular the aponeuroses of the three muscles join while coursing across the front of the rectus, leaving the latter's posterior surface in direct contact with the transversalis fascia. According to the same accounts the sheath is deficient in the thoracic region, the muscle resting on the costal cartilages posteriorly, covered anteriorly by the external oblique aponeurosis.

Actually irregularities in the composition of the laminae are common, with the result that the position, the outline, and the

¹ Contribution No. 307 from the Anatomical Laboratory of Northwestern University Medical School.

distinctness of the linea semicircularis are subject to considerable variation. Yet, in but few articles in the anatomical literature have the conventional statements regarding the constitution of the sheath been either questioned or refuted. Mackay disagrees with the statement that the linea semicircularis marks the lower limit of the posterior wall of the rectus sheath, stating that the posterior lamina is continued downward beyond the linea as a comparatively thin but distinct layer. Walmsley believes that "the external oblique aponeurosis is not a true part of the rectus sheath but a superficial covering of it." Walmsley recognizes the presence of an internal fascial investment distinct from the aponeurotic components of the sheath proper. He also illustrates possible variations in the sheath dependent upon the following arrangements of layers: (a) splitting of the transversus aponeurosis, the aponeuroses of the internal and external oblique joining the anterior lamina thereof; (b) passing of the external and internal oblique aponeuroses in front of the rectus, the transversus aponeurosis alone passing behind; (c) transversus and internal oblique coursing behind the rectus muscle, the external oblique alone passing in front.

MATERIAL AND METHODS

Dissections of the rectus sheath were made on one side of fifty-six cadavers. Measurement of height of the linea was taken, perpendicular to a line drawn through the pubic crests; the height of both the medial and lateral attachments of the arch was recorded, since the latter, singly, would give no indication of the point of insertion of the aponeurotic fibers.

The aponeurotic composition of the separate laminae was studied by dividing the sheath into gross segments; the course of the fibers could then be observed by separating the aponeuroses medially as far as possible, loosening the component bands at their point of decussation; thus mobilized they could be drawn back and forth much as one would move the interdigitating fingers of the clasped hands.

OBSERVATIONS AND DISCUSSION

The linea semicircularis in the fifty-six cadavers dissected for this study reveals wide variation in position (fig. 1).² The summit of the arch formed by the fold may be almost as high as the umbilicus or so close to the pubic crest that the aponeurotic defect resembles an enlarged foramen for transmission of the inferior epigastric vessels. The linea semicircularis may cross the posterior wall of the sheath in an almost horizontal plane, or arch upward or downward. Downward inclination is the common arrangement, the medial point of insertion being at a lower level than that of the origin in 63% of the cases; in 27% the insertion is higher than the origin, while in 10% the extremities are on the same horizontal plane. The highest point of origin of the linea above the pubic crest is 13.0 cm., the lowest 4.0 cm.; the average height is 8.0 cm. Among those cases in which the insertion is lower than the origin there are eight in which the band inserts into the pubic crest; one of these eight cases exhibited maximal vertical distance of 7.0 cm. between medial and lateral extremities.

Inferior to the linea semicircularis the rectus muscle, invested by its own thin layer of fascia, rests upon the preperitoneal connective tissue (Anson and McVay, '38 a, '38 b); it does not lie upon the transversalis fascia proper. The transversus aponeurosis and the main part of the transversalis fascia pass anterior to the muscle; however, although the fascia does continue with its aponeurosis (fig. 2, at a), a leaf, derived from the main layer (fig. 2, at b) splits to invest the muscle (fig. 2, at c), then continues to the linea alba. Assum-

²In figure 1 measurements are grouped in the category of the nearest $\frac{1}{2}$ cm. The dotted lines represent actual variations in the course of lineae semicirculares which have a similar point of lateral origin; the 5-cm. and 10-cm. heights were chosen as examples, comparable variations being found at the other levels. The measurement of height were made from a transverse line drawn through the pubic crest to the point of lateral limit of the linea semicircularis.

In figures 3 to 5 the muscle has been removed inferiorly to expose the posterior lamina of the sheath, but, for reasons of clarity, has been included in all the figures (3b, 5b, etc.) depicting sections. In examination of specimens of the type illustrated in figures 6, 7 and 8, aponeurotic fibers are sometimes discoverable only through the use of light placed behind the lamina (i.e. through transillumination).

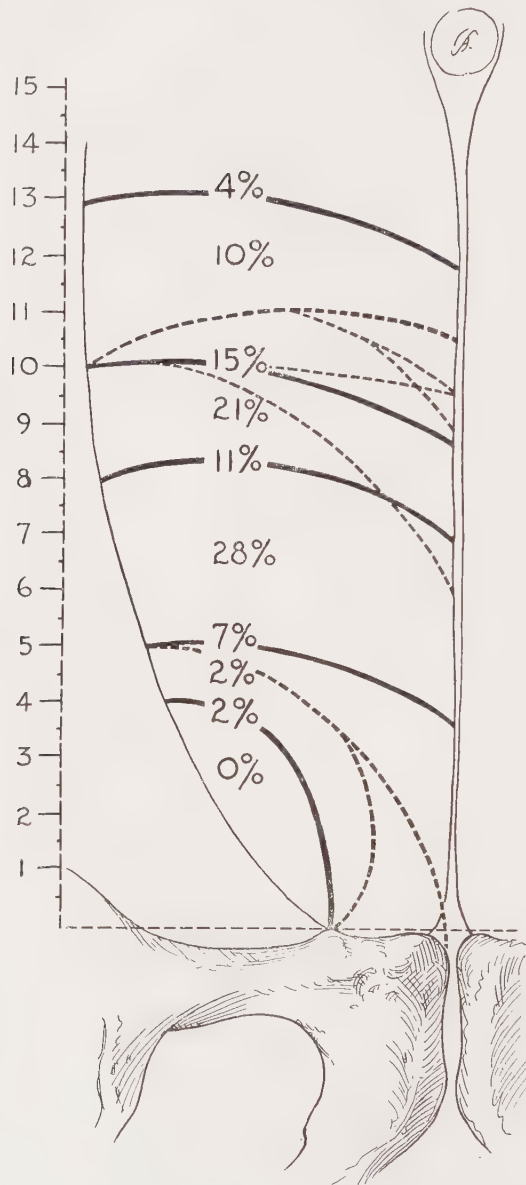


Fig. 1 Diagram illustrating the variation in height (in centimeters) of the linea semicircularis; percentage-occurrence is recorded for each category; broken lines indicate differences in form (see footnote 2).

edly, this is the fascial stratum described by Walmsley (see above).

In two types of cases it appears, upon initial examination, that the layer situated posterior to the rectus muscle (caudal to the linea semicircularis) is the transversalis fascia. In the first group the hypogastric part of the preperitoneal connective tissue is a membranous nonfatty layer which resembles a fascia; laterally, however, it is traceable into the fatty preperitoneal connective tissue of the iliac fossa, of which stratum it is merely a locally altered portion. The occurrence of this type of preperitoneal connective tissue accounts for the widespread opinion that, on the lower abdomen, the transversalis

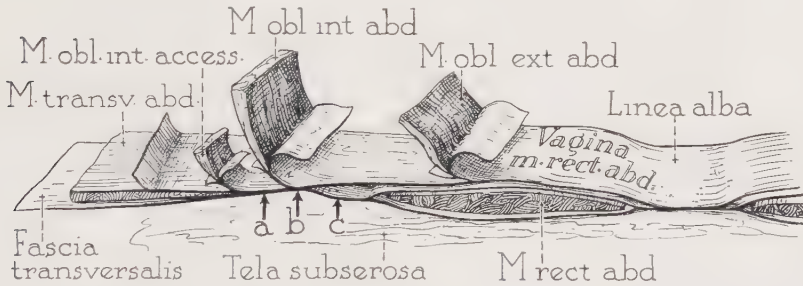


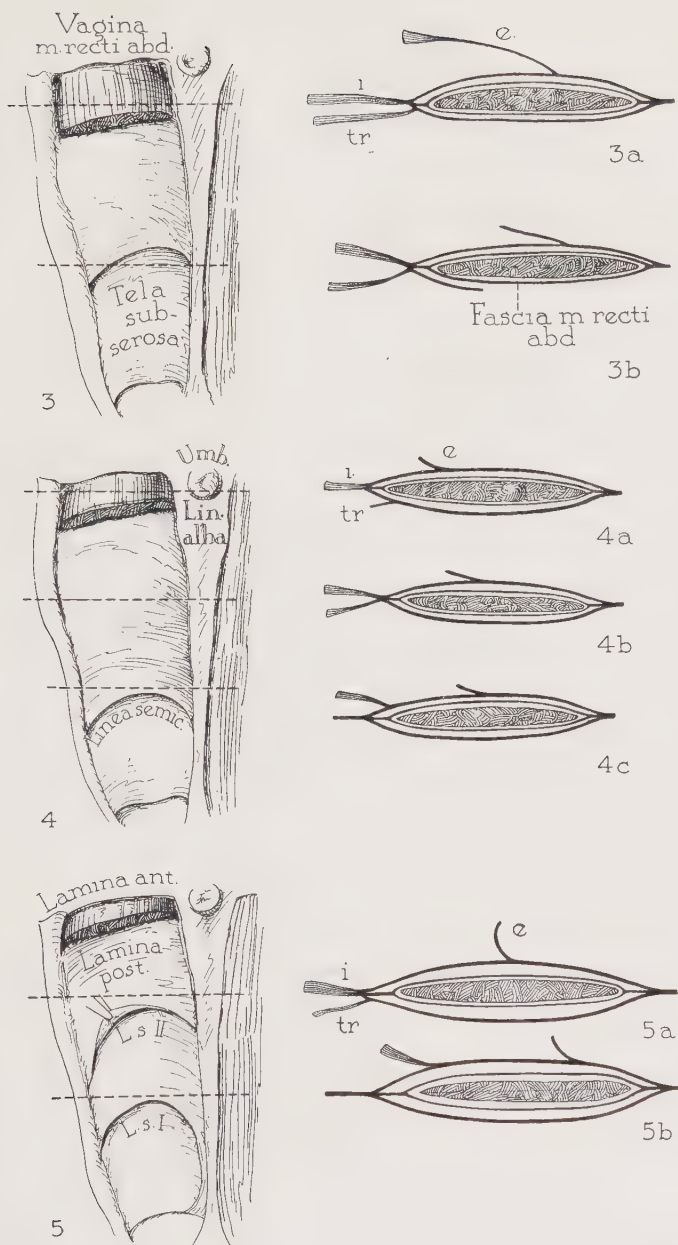
Fig.2 Segment of the rectus muscle, sheath and contributory aponeuroses, inferior to the level of the linea semicircularis. Showing the manner in which the transversalis fascia, fused with the aponeurosis (at a), gives off (at b) a thin fascial layer (at c) which splits to invest the rectus muscle.

fascia bounds the rectus muscle posteriorly. In the second group of cases, in addition to a well-defined linea semicircularis, there occur scattered aponeurotic fibers of the transversus aponeurosis in the area inferior to the linea; held together by transversalis fascia, these aberrant fibers may be mistaken for a fascia; but even in such cases the thin supporting fascial lamina does not go all the way to the pubic crest, but ends exactly with the lowermost aponeurotic fibers (thickened, above L.s.I in fig. 8).

Without exception in the standard text-books of anatomy it is stated that at the linea semilunaris the aponeurosis of the

internal oblique divides into an anterior and a posterior lamina, joined anteriorly by the external oblique aponeurosis and posteriorly by the transversus aponeurosis; this has come to be regarded as a classical arrangement.³ But upon theoretical grounds, as stated by Walmsley, several other arrangements of lamellation are possible in the area cranial to the semicircular line of Douglas: (a) the transversus (not the internal oblique) may divide to enclose the rectus muscle; (b) none may divide, the external and internal oblique passing in front of the rectus, the transversus behind it; (c) or, no layer dividing, the external aponeurotic layer may pass in front, the intermediate and the deep layers coursing behind the muscle. In the authors' dissections, however, no cases have been encountered in which decussation of aponeurotic fibers failed to occur; in other words, representatives of the above categories (b) and (c) were wanting. But while it is true that fusion or interdigitation of fiber-bands occurs in all of the current dissections, yet, remarkably, in the authors' series of fifty-six cadavers, only two cases present the classical arrangement. In forty-three of the fifty-six cases, it is the transversus aponeurosis that divides at the linea semicircularis, the entire aponeurosis of the internal oblique passing anterior to the rectus muscle (fig. 5). In the remaining eleven cases both the internal oblique and transversus aponeuroses give aponeurotic fibers to the posterior lamina of the sheath at the linea semicircularis and both of them contribute to the anterior lamina (fig. 3), by decussation of fibers; the aponeurotic fibers of the two muscular strata interdigitate (fig. 3a) superior to the level of the linea semicircularis, abruptly change arrangement inferiorly (fig. 3b) all passing anterior to the muscle.

³ In the study by Chouke ('34-'35), variation in splitting of the internal oblique aponeurosis is presented in relation to the occurrence of the accessory internal oblique muscle. The article reviews the various attempts to explain the sudden change in constitution of the sheath at the semicircular line. Cullen and Brödel ('37) presented a detailed account, superbly illustrated, of the structure of the rectus muscle—its fascicular arrangement in relation to the course of the vessels and nerves; the article does not include however, a consideration of variation in the constitution of the rectus sheath.



Figs. 3 to 5 Specimens illustrating variation in structure of the rectus sheath. The sections shown in figures 3a, 3b, etc., record the aponeurotic contributions to the sheath at the levels indicated by lines (see footnote 2). Abbreviations: L.s. I, L.s. II, linea semicircularis prima, secunda, respectively; e, m. obliquus externus abdominis; i, m. obliquus internus abdominis; tr, m. transversus abdominis.

There may be a secondary linea semicircularis more superiorly placed than the primary one; the secondary linea owes its existence to groups of fibers in the posterior lamina which abruptly change their course. Thus, near the umbilical level, the fibers of the internal oblique divide (fig. 5a); nearer the pubis (fig. 5b) the transversus abdominis divides to invest the muscle, the change from one arrangement to the other occurring within such a narrow area of the posterior lamina that a secondary linea semicircularis is produced (fig. 5). A secondary linea occurs in twenty cases of the total series studied, or in 47% of the group in which the primary linea semicircularis is formed by the divided transversus aponeurosis. The maximum distance between lineae, in the authors' case is 5.3 cm.; the minimum is 0.8 cm. while the average interdistance is 2.7 cm.

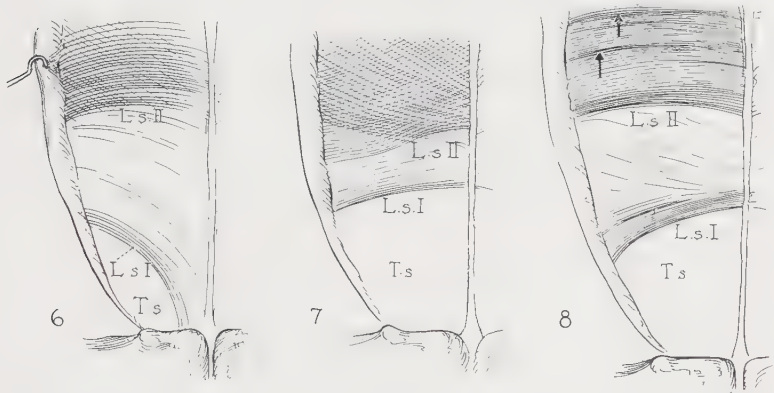
In twenty-three cases (53% of the latter group) there is no secondary fold (fig. 4) owing to the fact that the aponeurosis of the internal oblique contributes fibers to the posterior lamina very gradually. Thus, at the level of the umbilicus (fig. 4a), only the aponeurotic fibers of the internal oblique divide to enclose the muscle; at an intermediate level (fig. 4b), both internal oblique and transversus abdominis exchange fibers in such a way as to contribute equally to each lamina by decussation, while, in the succeeding zone (fig. 4c), the transversus abdominis divides to enclose the muscle, the fibers of the internal oblique joining the anterior one of the two laminae.

In specimens in which a secondary linea is present, considerable difference may exist in the appearance of the posterior lamina. Three specimens may be used as examples of the several arrangements: (1) along a stripe situated just above a secondary linea and in a band above a definite primary linea, fibers of both the internal oblique and the transversus decussate, while in the area between the lineae few fibers of the transversus abdominis are sent to the posterior lamina (fig. 6); (2) decussating fibers of the internal oblique and the transversus abdominis contribute to both the anterior and the posterior lamina except in the area between the lineae, where

the transversus alone forms a thin posterior lamina (fig. 7); (3) between lineae the posterior lamina contains only sparse aponeurotic fibers of the transversus added to the subjacent transversalis fascia (fig. 8).

Partial or incomplete lineae also may occur, formed of bands of transversus fibers which are added to the regular posterior lamella of the divided internal oblique (fig. 8, at arrows).

Where the rectus muscle crosses the costal margin, the sheath, as a complete envelope, must terminate, since the internal oblique muscle and its aponeurosis gain a costal attachment. The anterior lamella of the internal oblique aponeurosis



Figs. 6 to 8 Specimens illustrating variation in composition of the posterior lamina of the rectus sheath. Arrows indicate incomplete lineae semicirculares. Striations indicate the course of aponeurotic fibers, as determined by transillumination. Abbreviations as in figures 3 to 5.

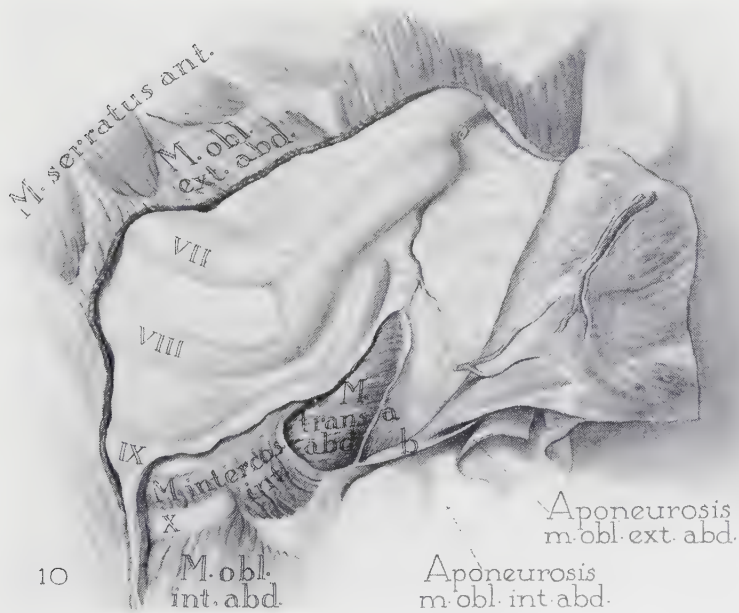
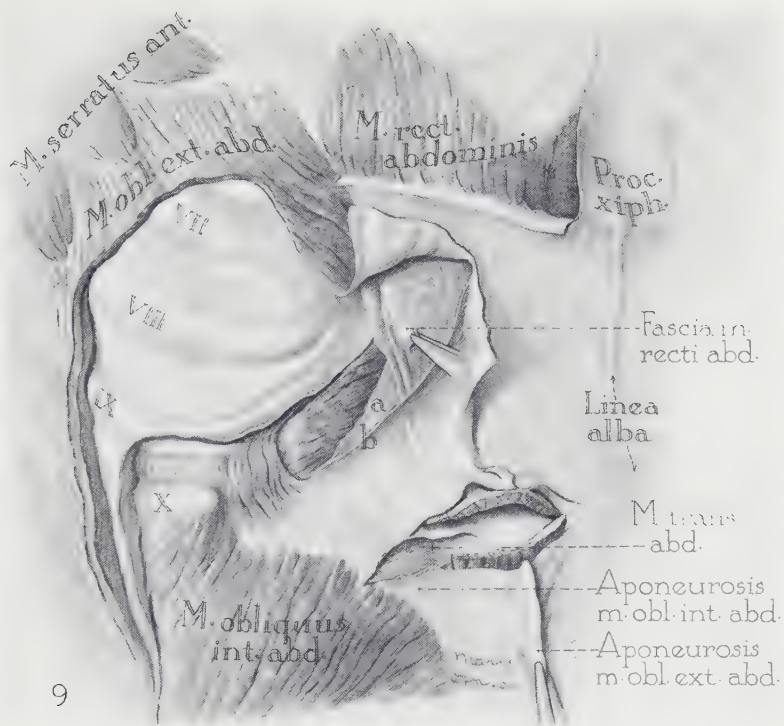
ends as a sharp line that passes superomedially (fig. 9, at b), continuing the direction of the fascicles in the internal oblique muscle. Above this line, the rectus muscle and its fascia are covered only by the external oblique aponeurosis; here there is no true delimiting sheath at the lateral margin of the rectus muscle. The posterior lamella of the internal oblique aponeurosis (fig. 9, at a) ends superiorly in less abrupt manner, its aponeurotic fibers being embedded in the fascial layer that covers the external surface of the transversus abdominis

muscle (fig. 10, at a). There is, therefore, a certain similarity between the linea semicircularis inferiorly and the termination of the rectus sheath superiorly, since each represents the end of an aponeurotic stratum.

As described above, the fascia of the rectus muscle, inferior to the linea semicircularis, is the medial slip derived from the transversalis fascia. In those instances in which scattered aponeurotic fibers occur inferior to the linea semicircularis, they are supported in a fascial layer which is continuous with the fascia transversalis, but separate from the rectus fascia. Superior to the level of the linea, where the rectus sheath is formed by the split transversus aponeurosis, the rectus fascia arises laterally in the angle formed by this division; medially, at the linea alba, the rectus fascia is attached in the angle where the lamellae again fuse; a comparable arrangement exists in those cases in which the internal oblique, not the transversus, aponeurosis divides. When the sheath is formed by a splitting of both the internal oblique and the transversus abdominis aponeurosis, it is possible to demonstrate continuity of the rectus fascia with the fascial laminae situated between the internal oblique and transversus muscles, through the interspaces existing between groups of decussating fibers. Above the level of the rectus sheath proper, i.e. above the point where

Fig. 9 Superior portion of the rectus sheath with contributory abdominal layers. The pectoralis major has been removed, the external oblique remains only marginally; the investing fascial layers have been removed up to the point where muscle gives way to aponeurosis. The rectus abdominis is exposed by removal of the costal part of the anterior lamina; its fascia (lifted by forceps) is separable from the sheath. It is shown that the superior part of the anterior lamina consists of the aponeurosis of the external oblique, the internal oblique not being distinguishable at this level, while, inferior to the costal margin, the same lamina is of double origin (at the two incisions). The aponeurosis of the internal oblique, at the costal extremity, divides early, leaving the lateral portion of the sheath unoccupied by muscle at the costal level (upper incision). The inner lamella of the internal oblique aponeurosis (incised at a) rests upon the transversus abdominis muscle; the outer lamina, on the contrary, ends abruptly (at b).

Fig. 10 The rectus muscle is here turned aside to expose the internal oblique. The posterior lamella is heavy along the costal margin (at a), but diminishes in prominence as it extends upward toward the xiphoid process, gradually merging with the thin fascial covering of the transversus abdominis muscle.



the divided internal oblique aponeurosis ends, the rectus fascia is the medial continuation of another slip, similar in nature, that arises from the fascial lamina on the deep aspect of the external oblique muscle and its aponeurosis. The marginal bands which, according to some authors, fasten the medial and lateral margins of the rectus muscle to the angles of the sheath (subdividing the cavity within the rectus sheath into anterior and posterior compartments) are doubtless the attachments of the rectus fascia at the angles of the sheath.

The external oblique aponeurosis joins that of the internal oblique very near the linea alba in the lower abdomen, midway between the linea alba and the linea semilunaris in the upper abdomen (figs. 9, 3a, 4b, and 5a). But, although this junction affects the external and internal oblique layers in the anterior lamina, complete fusion occurs; contrary to the opinion of Mackay, the present authors find that the external oblique is part of the true sheath.

CONCLUSIONS

There is a wide variation in the position of the semicircular line; the summit of the arch may be almost as high as the umbilicus or so close to the pubic crest that the defect resembles a large foramen for transmission of the inferior epigastric vessels.

The usual statement, that above the linea semicircularis the rectus sheath is composed of a divided internal oblique aponeurosis supported anteriorly by the external oblique aponeurosis and posteriorly by the transversus, does not apply in most instances: in only two of the fifty-six bodies studied was this arrangement found to exist through the area superior to the semicircular line. In the remaining specimens one of the following arrangements obtained: (1) the transversus abdominis aponeurosis divided at the linea semicircularis and continued medialward either with a gradual decussation of fibers or abruptly with the formation of a secondary linea semicircularis; or (2) both internal oblique and transversus abdominis aponeuroses divided at the linea semicircularis,

sending decussating fibers to the anterior and posterior laminae of the sheath.

At its superior extremity, the rectus sheath terminates abruptly in an oblique line which represents the point at which the internal oblique ceases to contribute to the sheath.

In addition to the aponeurotic sheath, the rectus muscle is encased in a fascial sheath whose derivation varies at the different levels.

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SEX DIFFERENTIATION IN TRIPLOID NEWTS (TRITURUS VIRIDESCENS)

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THREE TEXT FIGURES AND THREE PLATES (THIRTY-FIVE FIGURES)

INTRODUCTION

Triploid larvae of the newt, *Triturus viridescens*, occur occasionally in nature. Since these exceptional individuals can be identified in life, by chromosome counts in amputated tail tips, it is possible to follow their development in the laboratory and to investigate the effects of the increase in chromosome number.

During the winter 1937-1938, four triploid larvae of *Triturus viridescens* were raised in the laboratory and preserved during or immediately after metamorphosis (Fankhauser, '38b). In other species of newts, sex differentiation has been shown to take place shortly before metamorphosis; the condition of the gonads of the four triploids is therefore particularly important, since it should tell us whether or not the presence of a third set of chromosomes interferes with the normal process of sex determination, as it does in insects and in some dioecious plants. This approach to the study of the mechanism of sex determination in amphibia is all the more desirable, because conclusive cytological evidence is lacking and breeding experiments with hermaphroditic frogs and sex-reversed toads have given contradictory results.

The observations on the gonads of the four triploids have been briefly summarized in a preliminary note ('38c). A detailed account will be given in the present paper. Inasmuch as the normal process of sex differentiation in this species has not been fully described before, the normal development

of the gonads, from the first beginning of differentiation to metamorphosis, will be considered first. This account is based on the unpublished investigations of Mr. L. D. Fenninger ('38).

The appearance of the four triploid animals at the time of fixation has been described previously ('38b). They were preserved in Michaelis' fluid and sectioned at 15 micra, from the base of the tail forward. In this way the sections become so oriented that the left side of the animal appears to the left in all the photographs. The sections were stained with Harris' acid haemalum and Orange G. The photomicrographs were taken with a Zeiss planachromate 40X and a 5X compensating ocular. In reproduction, the magnification was reduced to 220X (plate 1) and 198X (plates 2 and 3).

THE NORMAL PROCESS OF SEX DIFFERENTIATION

The normal development of the gonads follows closely the descriptions given for other species of salamanders by Abramowicz ('13, *Triton taeniatus*), Burns ('25, *Amblystoma punctatum*), and McCurdy ('31, *Triturus torosus*). Before the age of 11 weeks (at 20° to 22°C.), the gonads are small and undifferentiated, similar to those shown in figure 4. All the germ cells have large, lightly staining, and irregularly lobed nuclei. In some of the 11-week-old larvae, groups of small, more or less spherical nuclei appear (fig. 5). These nuclei are the first indication of differentiation in female direction, because spherical nuclei of various structural types are typical for oogonia and oocytes in later stages and completely absent in young testes (figs. 13 and 16).

At the age of 12 weeks, the gonads clearly fall into two classes. In one group, all the germ cells still possess the polymorphic nuclei that are characteristic for the undifferentiated gonad (fig. 6). In the other class, the oval or spherical nuclei have increased in number and, to some extent, in size (fig. 7), but show a similar reticular structure as before.

In 13-week-old animals, the young ovaries (fig. 9) are still more definitely characterized by the presence of a small cavity

in some of the sections, and by the appearance of larger spherical nuclei with the typical "spireme-like" structure described particularly by Burns. These cells probably are small oocytes in early stages of the prophase of meiosis (Rodgers and Risley, '38, p. 124). The nuclei of young oocytes in the early growth period show a similar structure (figs. 14, 15, 17, 18), and similar cells have been described as oocytes in ovaries of adult salamanders (Fischer, '35). The other type of gonads, presumably young testes, resembles closely undifferentiated gonads, until the beginning of metamorphosis (figs. 8 and 10).

In larvae 14 weeks old, the gonads are considerably larger (figs. 10 to 12). In the ovaries, the cavity is clearly marked, and the small oocytes have increased in size and number. Undifferentiated germ cells, with pale, irregularly lobed nuclei, are still present and persist through metamorphosis (fig. 17). Similar cells are also found in the wall of mature ovaries.

At the beginning of metamorphosis (about 16 weeks from fertilization), the ovaries contain growing oocytes which begin to bulge out into the cavity (figs. 14 and 15). At the periphery, nests of germ cells (oogonia and, predominantly, very young oocytes) are present. The testes are solid structures, with germ cells scattered throughout the cross section (fig. 13). The nuclei of these spermatogonia are still polymorphic, but in the majority of the nuclei the lobes are broader and more rounded in contrast to the narrower, more pointed lobes of the undifferentiated germ cells (figs. 20 and 21). This change in the shape of the nuclei of the germ cells seems to be the first distinguishing feature between undifferentiated gonad and testis.

Toward the end of metamorphosis (about 18 weeks from fertilization), the testis still presents a similar picture (fig. 16), while the ovary has grown considerably (figs. 17 and 18). This increase in size is connected primarily with growth of

the larger oocytes which almost completely obliterate the ovarian cavity.

At the time of metamorphosis, ovary and testis are therefore clearly differentiated by the following features (cf. Burns, '25):

The ovary possesses a small central cavity, nests of oogonia and small oocytes with spherical nuclei at the periphery, and growing oocytes protruding into the central cavity; undifferentiated germ cells with polymorphic nuclei are still present in considerable number. The testis is a solid structure, with germ cells distributed throughout the cross section. All the germ cells (spermatogonia) have broadly lobed nuclei which differ slightly from the polymorphic nuclei of undifferentiated germ cells.

Reconstructions show that the testes are shorter than the ovaries (ca. 950 to 1000 micra against 1650 to 2450 micra, figs. 1 to 3). Their anterior end is usually located near the tenth spinal ganglion, while the ovaries may extend beyond the ninth ganglion. The left ovary is frequently shorter than the right, but may be wider and slightly more advanced in its differentiation.

THE GONADS OF THE FOUR TRIPLOIDS

The youngest triploid (no. 20) was fixed at the age of $15\frac{1}{2}$ weeks, shortly after the beginning of metamorphosis. The gonads are typical testes, normal in every respect, as the comparison of figures 19 with 13 and 16 shows. Graphic reconstructions (fig. 1) demonstrate that the testes are of about the same length as in the controls, but slightly wider.

The second triploid (no. 5) was fixed toward the end of metamorphosis, at the age of 17 weeks. Three representative sections through the gonads are shown in figures 25 to 27. A comparison with corresponding sections through the ovaries of a control (figs. 22 to 24) at once demonstrates that the gonads of the triploid are ovaries; a small central cavity is present, and germ cells with spherical nuclei (young oocytes) are visible in figures 25 and 27. However, the undifferentiated

germ cells with polymorphic nuclei predominate (fig. 26), and the cross section of the ovary as a whole is definitely smaller than normal. The last-mentioned difference is also clearly brought out by reconstructions (fig. 2).

The gonads of the third and fourth triploids (nos. 10 and 11), fixed at the age of 18 weeks, present a similar picture

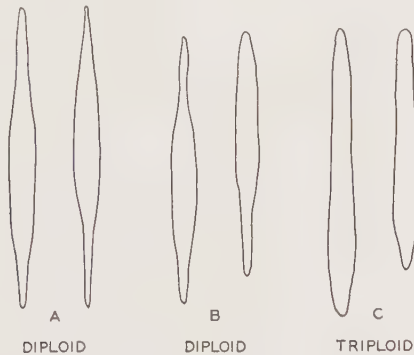


Fig.1 Reconstructions of testes of three animals in metamorphosis. A, diploid no. 4, 16 weeks. B, diploid no. 6, 18 weeks. C, triploid no. 20, 15½ weeks.

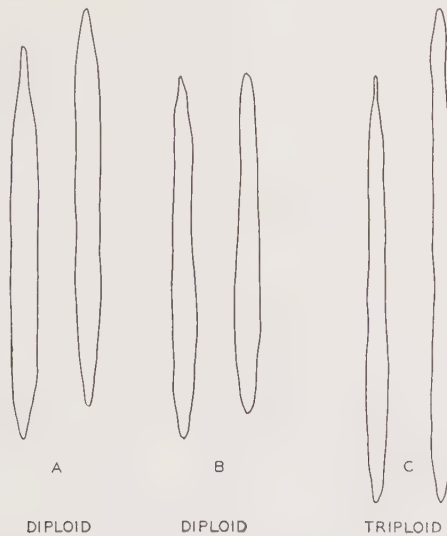


Fig.2 Reconstructions of ovaries. A, diploid no. 1, 17½ weeks. B, diploid no. 13, 16 weeks. C, triploid no. 5, 17 weeks.

(figs. 30 to 32, and 36 to 38). The gonads are rudimentary ovaries, with a central cavity and a small number of young oocytes. The undifferentiated germ cells are more numerous, particularly in the left gonad of the fourth triploid (figs. 36 to 38). Oocytes in early growth stages are entirely absent. The reconstructions of figure 3 also demonstrate the reduced size of the triploid ovaries.



Fig. 3 Reconstructions of ovaries. A, diploid no. 12, 18 weeks. B, triploid no. 10, 18 weeks. C, triploid no. 11, 18 weeks.

A more accurate basis for comparisons is given by the actual number of germ cells present in the ovaries of different animals. The various types of germ cells were counted in the left ovary of the triploid no. 5 (figs. 2C and 25) and of three diploid animals, 11, 14 and 17½ weeks old (figs. 5, 12 and 22). Table 1 shows that the total number of germ cells in the triploid ovary is only about one-third of the normal number. Cells with spherical nuclei are still in the minority and should probably be classified as very young oocytes, while

in the control small oocytes with typical prophase nuclei are predominant.

A more detailed investigation would be necessary to establish with certainty the significance of all the different

TABLE 1

Number of undifferentiated germ cells, oogonia, and oocytes in left ovary of two larvae and two animals at end of metamorphosis

TYPE OF NUCLEUS	TYPE OF GERM CELLS	LARVA 11 WEEKS	LARVA 14 WEEKS	DIPLOID NO. 1 17½ WEEKS	TRIPLOID NO. 5 17 WEEKS
Small, lobed (found at posterior end of gonads only)	undifferentiated	19	18	—	—
Large, lightly stained, irregularly lobed (polymorphic)	undifferentiated	68	100	206	132
Small, oval or spherical, with reticular structure	oogonia	62	249	—	—
Small to medium, spherical, chromatin in tightly massed threads (poor fixation of leptotene and zygotene stages of meiosis?)	very young oocytes	—	186	56	69
Medium, spherical, with prophase structure (probably early pachytene)	young oocytes	—	106	315	1
Larger, spherical, with more diffuse chromosomes	oocytes in early growth stage	—	9	11	—
Mitoses		—	3	1	—
Degenerating nuclei		—	—	4	4
Total		149	671	593	206

types of germ cells that are present in the young ovary. However, a comparison of the numbers of different cell types found in the left ovary of the three diploid animals of different ages suggests strongly that the cells with spherical nuclei and

massed chromatin threads represent an intermediate stage between the two adjacent types; probably they are oocytes in the earliest stages of meiotic prophase.

DISCUSSION

Of the four metamorphosed, triploid newts examined, one contains normal testes, while the other three possess rudimentary ovaries. Although there is some variation in the condition of the ovaries in the three cases, their appearance is still sufficiently uniform to be characteristic for the triploid females. Triploidy, therefore, affects the female sex only, while the male sex appears to be normal.

In animals with a definite sex chromosome mechanism, triploidy has no effect on the homogametic sex which normally possesses two X-chromosomes, while the heterogametic sex, with a single X-chromosome in the diploid complement, is changed to an intersexual condition. In *Drosophila* and *Carausius*, triploid females are therefore normal, but no normal triploid males are produced. In moths, on the other hand, triploid individuals are either normal males or intersexes, because the female sex is heterogametic (Bridges, '21; Goldschmidt and Pariser, '23; see also Vandel, '37).

In triploid newts, it is also the female sex that is affected. However, it would be premature to conclude from this that newts actually have the same type of sex determination as moths. It is impossible to determine whether the presence of rudimentary ovaries in three of the four triploids indicates intersexuality, or merely a retardation in the development of the gonads, caused perhaps by some general, physiological effects of triploidy. It should be mentioned, however, that the triploid intersexes produced by back-crossing hybrids between different species of moths are all characterized by rudimentary ovaries, although they vary greatly in the appearance of the other sexual characters (cf. Goldschmidt, '31).

Cytologically, sex chromosomes have not been demonstrated conclusively in any amphibian, so far. In *Triton palmatus*, a

detailed study of the morphology of individual chromosomes during cleavage revealed the existence of a slightly heteromorphic pair in three eggs out of thirteen (Fankhauser, '34). There is no definite indication, however, that this pair represents sex chromosomes.

Breeding experiments with hermaphroditic frogs appear to demonstrate male heterogamety (Crew, '21; Witschi, '23, '29). In accordance with these observations, the diploid parthenogenetic frogs that can be reared through metamorphosis would all be expected to be females. In recent experiments with *Rana nigromaculata* and *Rana japonica*, Kawamura ('39) found, among twenty-three parthenogenetic young frogs, fifteen typical females, three individuals with abnormal or degenerating ovaries, two which were "almost transformed into males," two with rudimentary testes, and one male. According to his interpretation, the individuals with testes are genetic females that have undergone sex reversal, caused perhaps by abnormalities in other organs. A similar explanation was suggested by Parmenter ('25) for the occurrence of males in Loeb's material, although in these cases over-ripeness of the eggs is indicated as the possible cause of sex reversal.

The results of breeding experiments with sex-reversed male toads are also not entirely conclusive. Harms ('26) obtained both males and females, while Ponse ('31) found exclusively males, among 400 individuals from several batches of eggs, a fact which suggests a homogametic condition of the male. It is possible, of course, that frogs and toads possess different sex chromosome mechanisms, just as male and female heterogamety may be found in closely related species of fishes.

Under these circumstances it would be interesting to study the effects of triploidy on sex in anurans. Kawamura ('39) reported one case of probable triploidy among his parthenogenetic frogs. The gonads of this individual are rudimentary testes. If the triploid condition of this animal is confirmed, the case would offer good evidence for the suggestion that

frogs and salamanders may differ in their sex chromosome mechanism.

Still more recently, an unexpected confirmation of the observations reported in this paper was received from Sweden. In the course of an investigation of spermatogenesis in *Triton taeniatus*, Böök ('40) found an individual with triploid testes. The testes and the other reproductive organs were entirely normal in appearance. The remaining parts of the animal had not been preserved but were presumably also triploid. This discovery of a sexually mature triploid male supports the conclusion presented here that the effect of triploidy on sex is not the same in newts as it is in *Drosophila*. This seems at first disconcerting in view of the fact that the only haploid newt that has so far been raised beyond the stage of sex differentiation proved to be a female (Fankhauser, '38a). The femaleness of this haploid *Triton taeniatus* appeared to be in perfect agreement with the expected, and partly observed, consequences of haploidy in *Drosophila* and suggested the existence of a similar sex chromosome mechanism in both animals. It is, of course, not certain that the mechanism in amphibians conforms exactly to either one of the two modes of sex determination found among insects. Unfortunately, the investigation of this mechanism is greatly complicated by the fact that, in amphibians, genetic sex determination is relatively labile and may be influenced or even reversed by various secondary factors.

For a more satisfactory analysis of the effects of triploidy on sex in salamanders it will be essential to have a large number of triploid animals available, and to raise them to a stage where the reproductive organs of the male and female are more clearly differentiated. Both of these requirements have now been fulfilled. On the one hand, it is possible to induce triploidy in *Triturus viridescens* by cold treatment of unsegmented eggs (Fankhauser and Griffiths, '39; Griffiths, '40). On the other, a rather high natural frequency of tri-

ploidy was discovered among larvae of the two-lined salamander, *Eurycea bislineata*, which can easily be raised to a more advanced stage (Fankhauser, '39). The condition of the gonads in these two new groups of triploids is under investigation at present. The observations made so far appear to confirm entirely the results reported here.

SUMMARY

1. The normal process of sex differentiation in *Triturus viridescens* is described briefly. At the time of metamorphosis, ovaries and testes are clearly differentiated. The ovaries possess a small central cavity, groups of germ cells (oogonia and very young oocytes) at the periphery, and larger oocytes which protrude into the central cavity. The oogonia and oocytes are characterized by the spherical shape and the structure of their nuclei. Undifferentiated germ cells with polymorphic nuclei are also present. The testis lacks a cavity, the spermatogonia are scattered uniformly throughout the cross section of the gonad and have polymorphic nuclei with broad, rounded lobes.

2. Four triploid larvae were raised in 1937-1938 and preserved during or after metamorphosis. The gonads were examined in serial sections through the whole animals. The gonads of one triploid are typical testes, those of the other three are rudimentary ovaries which contain less than one-half the normal number of germ cells, with undifferentiated germ cells in the majority.

3. In newts, triploidy therefore affects the female sex only. Whether the rudimentary conditions of the ovaries of the triploids indicates intersexuality, corresponding to that found in triploid moths, or merely a marked delay in growth and differentiation of the ovaries, caused by general physiological effects of triploidy, cannot be decided until more and older triploid individuals have been studied.

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PLATE 1

EXPLANATION OF FIGURES

All figures on plates 1 to 3 are photomicrographs of transverse sections through gonads. The figures on plate 1 are magnified $220\times$, those on plates 2 and 3 $198\times$, with the exception of figures 20 and 21 which are magnified $396\times$.

4 Eleven weeks. Undifferentiated gonads. Germ cells have lightly stained, polymorphic nuclei.

5 Eleven weeks. First indication of female differentiation: appearance of small, spherical nuclei with reticular structure (oogonia).

6 Twelve weeks. Undifferentiated gonads (testes?). Germ cells as in figure 4.

7 Twelve weeks. Ovaries. Larger, oval or spherical nuclei with reticular structure (oogonia).

8 Thirteen weeks. Undifferentiated gonads (testes?). Increase in number of germ cells.

9 Thirteen weeks. Ovaries. Appearance of small cavities. At periphery, larger germ cells with spherical nuclei (young oocytes in early prophase of meiosis).

10 Fourteen weeks. Testis. Germ cells of same type as in undifferentiated gonad.

11 Fourteen weeks. Left ovary. Two larger oocytes with spherical and more lightly staining nuclei are visible, also two undifferentiated germ cells with pale, polymorphic nuclei. Central cavity larger.

12 Fourteen weeks. Left ovary of another larva. Similar to figure 11.

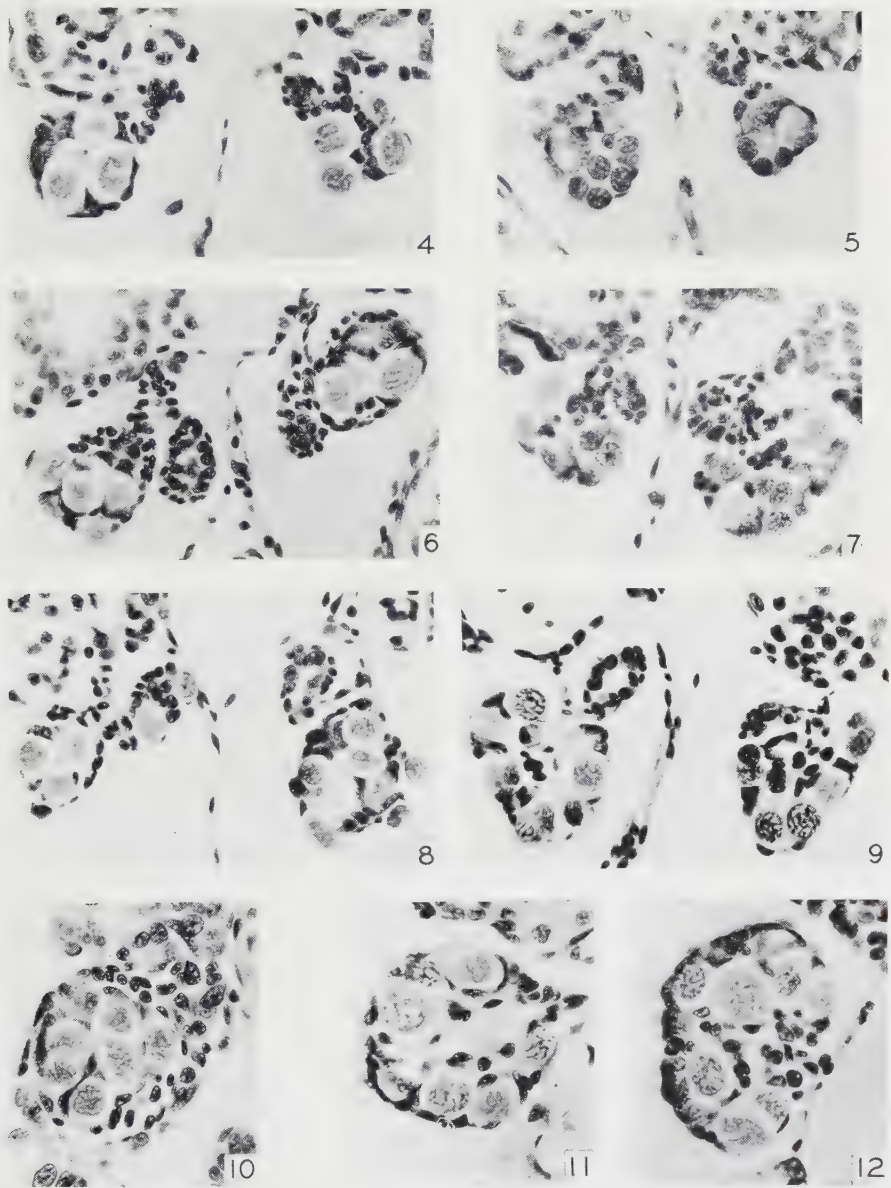


PLATE 2

EXPLANATION OF FIGURES

13 Sixteen weeks (beginning of metamorphosis). Left testis. Spermatogonia with polymorphic nuclei. Lobes of nuclei are broader and more rounded than in undifferentiated germ cells (cf. figs. 20 and 21).

14 Sixteen weeks. Left ovary. Two larger oocytes near center. Group of very young oocytes to left.

15 Same ovary. Larger oocyte in center, groups of very young oocytes above and to right. To left, three germ cells with polymorphic nuclei.

16 Eighteen weeks (end of metamorphosis). Left testis. Similar to figure 13.

17 and 18 Eighteen weeks. Left ovary, much larger than in figures 14 and 15. Growing oocytes are protruding into central cavity. In figure 17, larger groups of very young oocytes to right and in upper left hand corner. Undifferentiated germ cells with polymorphic nuclei are still present.

19 Triploid no. 20, 16 weeks. Left gonad. Typical testis.

20 Single undifferentiated germ cell from figure 6, enlarged to show polymorphic nucleus with irregular, narrow, and more or less pointed lobes.

21 Three spermatogonia from figure 13, enlarged to show broader, more rounded lobes of nuclei.

22 to 24 Diploid no 1, 17½ weeks (control for triploid no. 5). 22, left ovary, 23 and 24, right ovary.

25 to 27 Triploid no. 5, 17 weeks. 25, left ovary, 26 and 27, right ovary. Note small size of ovary, small number of young oocytes, and absence of larger oocytes. Many sections show undifferentiated germ cells with polymorphic nuclei only, as in figure 26.

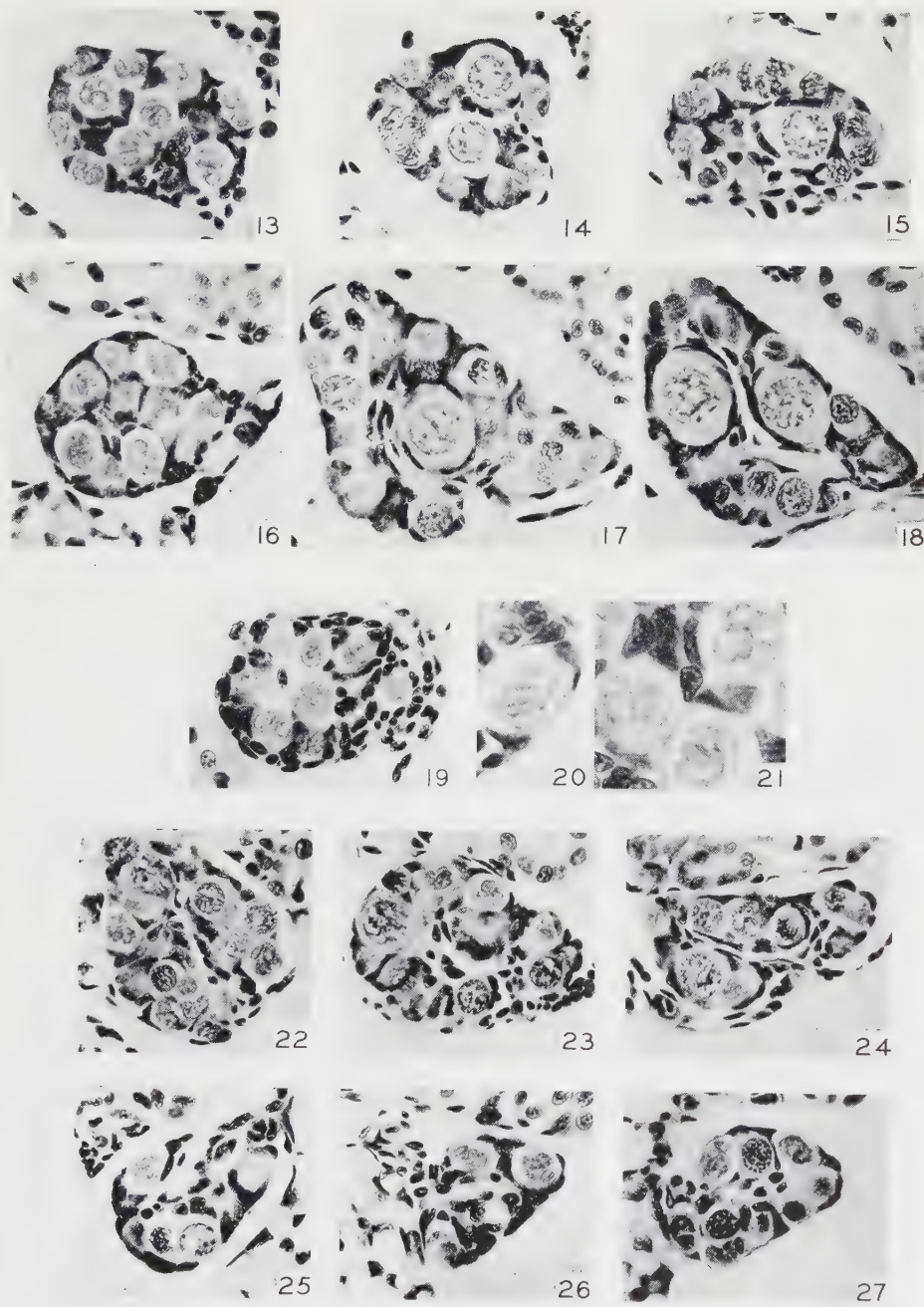


PLATE 3

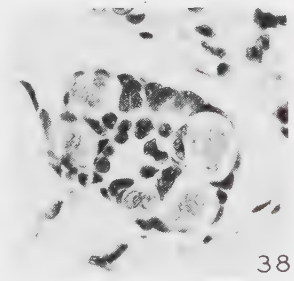
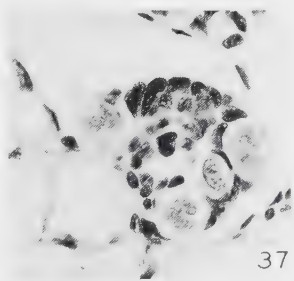
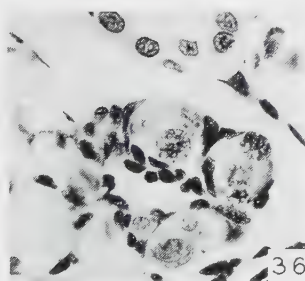
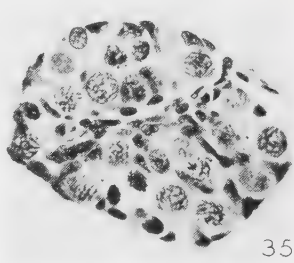
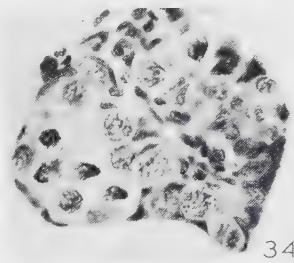
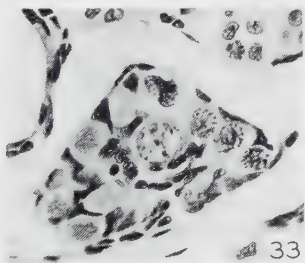
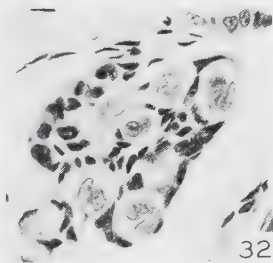
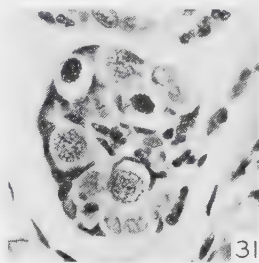
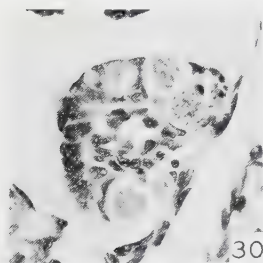
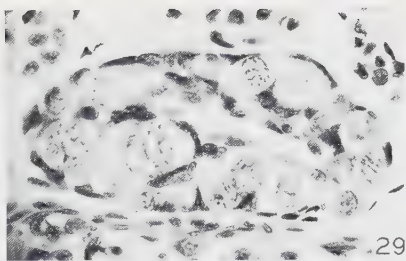
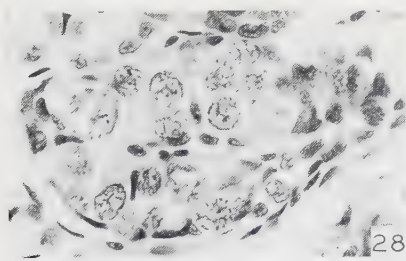
EXPLANATION OF FIGURES

28 and 29 Diploid no. 12, 18 weeks (control for triploids no. 10 and no 11). Left ovary.

30 to 32 Triploid no. 10, 18 weeks. 30 and 31, left ovary. 32, right ovary. Similar to figures 25 to 27. Undifferentiated germ cells with lobed nuclei are preponderant (figs. 30 and 32).

33 to 35 Diploid no. 2, 17½ weeks (control for triploids no. 10 and no. 11). Left ovary. Figures 28, 29 and 33 to 35 show the extremes of variation in the development of ovaries in controls of this age.

36 to 38 Triploid no. 11, 18 weeks. Left ovary. Few oogonia and young oocytes. Figures 37 and 38 resemble an undifferentiated gonad, except for presence of a central cavity.



EARLY HISTOGENESIS OF THE THYMUS IN THE WHITE RAT

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FOUR PLATES (SIXTEEN FIGURES)

The manner in which the thymus acquires its adult structure as a lympho-epithelial organ has long attracted the attention of investigators. The preponderance, although by no means all, of the evidence accumulated on this question over a period of years supports the belief held by Hammar ('10), Maximow ('09), Kingsbury ('15), and others, that the thymus reticulum and Hassall's corpuscles are derived from epithelium of the third pharyngeal pouch (in some animals from the third and fourth pouches) and that the so-called "thymocytes" are true lymphocytes of mesenchymal origin. Whether the thymus epithelium is entirely entodermal or arises in part from ectoderm is also a subject for debate. In at least certain cases—pig (Badertscher, '15), mouse (Crisan, '35), man (Norris, '38)—an ectodermal contribution from the cervical vesicle has been reported.

The development of the thymus in the rat has been studied cytologically to only a limited extent (Maximow, '09), although the morphology of the pharyngeal derivatives in this animal was described by Zuckerkandl ('03) and Rogers ('29). The present survey of the thymus in a number of rat embryos is an attempt to follow the early development of the epithelial and mesenchymal components of this organ and the fate of the cervical vesicle.¹

¹ The investigation was carried on in the Department of Histology and Embryology of Cornell University under the direction of Dr. B. F. Kingsbury, to whom the writer makes grateful acknowledgment. Thanks are also due to Dr. A. S. Woodman of Boston University School of Medicine for helpful advice during the preparation of this paper.

MATERIAL AND METHODS

The material consisted of serially-sectioned rat embryos ranging in age from the thirteenth day of development until birth, the stages being secured at approximately half-day intervals. A few sections of thymus from rats at 2 hours and at 6 hours after birth were also included. Since consideration of early stages was particularly desirable, the greatest number of specimens were of ages less than 18 days.

Of the 106 embryos studied, forty-three belonged to the collection of the Department of Histology and Embryology of Cornell University; these had been fixed in Bouin's or Carnoy's fluid, cut in paraffin at 10–15 μ , and stained with hematoxylin and eosin or hematoxylin and orange G. Sixty-three additional embryos obtained from timed rats purchased from The Wistar Institute were fixed in Helly's Zenker-formol, and serial paraffin sections of the cervico-thoracic region were cut at 6 μ and stained with Mayer's hematoxylin and azur II-eosin. On a few sections of thymus Masson's trichrome stain was used for identification of connective tissue elements.

OBSERVATIONS

As shown by Rogers ('29) the pharynx of the rat develops from the early foregut in a manner characteristic of other mammals, with the formation of three pairs of pharyngeal pouches. Pouch III gives rise to the thymus and parathyroid, the so-called complex III. No pouch IV ever appears in the rat.

The ultimobranchial body first arises as an evagination of the caudal wall of pouch III but early (12–13 days) acquires a separate opening from the pharynx. Although Zuckerkandl ('03) believed that this structure represented pouch IV in the rat, the writer prefers to assume, with Kingsbury ('15) and Rogers ('27), that the ultimobranchial body expresses a caudal growth tendency in the post-branchial region and is not homologous with a specific pouch. In the course of development this body becomes imbedded in the lateral lobe of the thyroid; whether, as claimed by Rogers ('27), it actually forms thyroid tissue could not be determined in this investiga-

tion. Ultimobranchial cysts such as occur so frequently in some animals were not found in any of the rat embryos examined.

The following material on the development of the thymus, and with it the parathyroid and cervical vesicle, has been divided arbitrarily into four parts, on the basis of certain general cytological changes; however it must be kept in mind that the processes involved are gradual and continuous, and subject to a considerable amount of individual variation.

Part I: 13- and 13½-day embryos. In an embryo of 13 days pouch III possesses a dorsal and a ventral diverticulum, of which the latter is the larger. The lumen of the pouch is still continuous with that of the pharynx through the ductus pharyngobranchialis. The greater part of the pouch is antero-posteriorly flattened by the third and fourth aortic arches, and its small lateral edge is fused with the ectoderm at the third branchial membrane. A well-defined cervical sinus has been formed; its lumen is divided by the projection of the third mesodermal arch into two smaller sinuses—one related to pouch II, the other to pouch III.

By the backward growth of the second mesodermal arch and lateral expansion of the post-branchial region, the cervical sinus becomes closed off (13 days 4 hours) and there are formed two vesicles associated respectively with pouches II and III and attached to the surface ectoderm by ductus branchiales II and III. A definite notch in the ectoderm for a time marks the point of closure of the sinus (fig. 1). The specific term "cervical vesicle" is commonly applied to the ectodermal vesicle related to pouch III (or to pouches III and IV when both are present) and will be so used in this discussion.

By 13 days 4 hours the whole of pouch III appears to have descended slightly, and the ventral diverticulum (thymus anlage) extends caudo-medially, with a tail-like portion bending around aortic arch IV. Prominent at the lateral side of this diverticulum is nerve XII.

At 13½ days pouch III has in most instances lost its connection with the pharynx; a remnant of the pharyngobranchial

duct, appearing as a small island of epithelium in the connective tissue (fig. 2), may be seen as late as $14\frac{1}{2}$ days. A lumen, round or crescentic, is present in the cranial part of the pouch. The large cervical vesicle is dorso-laterally apposed to complex III and lies in close contact with the ganglion nodosum of nerve X, with which, however, it is not at this stage actually fused. It is round or triangular, with a clearly-marked lumen, and until 14 days has a cellular connection with the surface ectoderm (remnant of ductus branchialis III).

Throughout this period all epithelial structures are cytologically much alike. The wall of the pharynx consists of a one- or two-layered cuboidal epithelium with indefinite cell boundaries and round or oval nuclei containing scattered chromatin. No marked difference can be seen between the cells of complex III and those of thyroid and ultimobranchial body, nor can a distinction at first be made between the epithelium which will become parathyroid and that of thymus. However by $13\frac{1}{2}$ days the nuclei of the parathyroid anlage are generally rather closely packed together. The surface ectoderm—one or two layers of flattened cells—reveals no fundamental difference from the entodermal elements. The cells of the cervical vesicle, however, are cylindrical and stain less deeply than those of the adjacent complex III.

As far as can be ascertained, the thymus anlage at this time is purely epithelial, no lymphocytes being present within its boundaries. The surrounding mesenchyme is dense and cell-rich but in it free wandering cells are extremely rare. Small blood vessels closely invest the epithelial derivatives of the pharynx but have not yet entered them. Mitotic figures are numerous in all tissues of this region.

Part II: 14- to 16-day embryos. In 14-day embryos complex III has begun to differentiate into its component parts. The dorso-medial parathyroid anlage can now be distinguished as a compact mass of cells with small nuclei and deeply-staining cytoplasm showing few cell boundaries. It is still closely united with the thymus. The latter organ, containing the narrow lumen of pouch III, consists of a head and a slender

tail which extends caudo-medially, bounded laterally by nerve XII and medially by aortic arch IV (fig. 2). The thymus cells are characterized by relatively pale cytoplasm and large round or oval nuclei with thin nuclear membranes and a sparse chromatin content; many of these cells are dividing. Neither parathyroid nor thymus has yet been invaded by blood vessels and connective tissue.

In this stage there appear significant alterations in the thymus (indicated in fig. 15). Certain of the epithelial cells undergo areolation, with the formation in their cytoplasm of colorless vacuoles, large and small; these may coalesce into larger spaces bridged by pale strands of cytoplasm. At this time also lymphocytes can first be recognized within the thymus in relatively small numbers, usually no more than four to seven in the entire organ. These cells are of the so-called large lymphocyte type. In material stained with azur II-eosin they may be distinguished by deeply-staining nuclei with heavy nuclear membranes and by cytoplasm of a clear blue color. Often in an imperfectly stained section such cells must be recognized by the nuclei alone, and it is possible that their number is greater than the above estimate, since only those identified beyond question have been enumerated. These lymphocytes, each surrounded by a pale area or "halo," are generally to be found in the cranial part of the thymus. Immigration from without is indicated by the position of some which occupy indentations of the surface of the thymus, although at this time free cells are still scarce in the surrounding mesenchyme. No lymphocytes are present in the parathyroid or cervical vesicle.

By 14½ days (fig. 3) the compact parathyroid has been partially set off from the thymus by connective tissue, and in some cases both organs have been invaded by capillaries. The thymus at this time extends almost to the pericardium, and irregular lobules have been formed by growth of the epithelial cells, whose pale cytoplasm shows continued vacuolation. Here and there in the organ lie large lymphocytes in slightly greater numbers; the somewhat looser mesenchyme also shows a small

increase in free cells. The cervical vesicle has now lost its close association with the ganglion nodosum and is located at the cranial pole of the thymus, either dorso-lateral or lateral, with nerve XII lying along its lateral aspect. It has been reduced in size, possibly by some cellular degeneration, as indicated by the presence of dark-stained granules and fragments within the epithelial cells, and by the loss of a small diverticulum which remains joined to the ganglion (see Rogers, '29).

In 15-day embryos (fig. 4) the large parathyroid, composed of cords of cells among abundant capillaries, lies dorso-medial to the thymus and is becoming elongated as if lagging in descent and being pulled out by growth tensions. Although bounded by a loose connective tissue capsule it still maintains a cellular connection with the thymus. The latter has increased in bulk and is supplied with numerous small blood vessels which enter through the deep connective tissue septa, exhibiting as yet no definite vascular pattern. There still remains a trace of the lumen of pouch III. The amount of vacuolation in the thymus is variable. The process is most evident in the cranial and caudal extremities of the organ; between these regions lie islands of unaltered epithelial cells. Lymphocytes are found in scarcely larger numbers than in the preceding stage. The small cervical vesicle always present in the cranial pole of the thymus consists of a single layer of pale, vacuolated, cuboidal or columnar cells surrounding a lumen which may contain a mass of coagulated material.

Except for changes in the relative positions of the pharyngeal derivatives the 15½-day stage differs but little from the preceding. The parathyroid lags still further behind the thymus but is joined with the latter by a strand of epithelial cells, the so-called "thymic cord." The large, much-lobulated thymus is now related cranio-medially to the lateral lobe of the thyroid and its base rests on the pericardium; nerve X lies along its lateral margin. A trace of the original lumen is variably present as an elongate cleft somewhere in the cranial half of the organ. The cytoplasm of the epithelial cells is much vacuolated and their nuclei lie far apart. Lymphocytes, mostly

large, are present within the thymus in somewhat greater numbers than before. The cervical vesicle resembles that of the preceding stage in structure and location.

At a stage of 16 days (fig. 5) the parathyroid may still be joined to the thymus; frequently, however, the thymic cord has ruptured, leaving a few of its cells attached to either thymus or parathyroid or both. Occasionally the cord is absent and a long-drawn-out strand of connective tissue marks its former position. The size of the thymus and its subdivision into lobules have increased. For the sake of convenience the right and left halves of the thymus may be referred to as lobes, although each arises from one of the third pair of pharyngeal pouches and not as a subdivision of a single primary structure; as the lobe of each side enlarges and elongates, the two begin to approach one another more closely in the midline. Small blood vessels entering the thymus laterally and medially appear to send their main branches up and down the longitudinal septa. Cytoplasmic vacuolation of the thymus cells has so increased as to form almost a reticulum, whose large pale nuclei are widely separated. Lymphocytes, large and small, are far more numerous than before; many are dividing. Although they are scattered throughout the thymus, the majority lie near the periphery. More lymphocytes are now present in the connective tissue but few appear in the blood vessels.

The cervical vesicle can be found in all embryos of 16 days, as in every previous stage (figs. 1-5); after this period it is variably present, persisting in a few instances as late as 19 days. It appears as a small circle of pale, vacuolated cells, wholly or partially imbedded in the thymus or lying cranio-lateral to it, surrounded by connective tissue. Even in late stages (figs. 8, 9) the cytology of this structure and its dorso-lateral relation to the thymus indicate its identity. A second type of vesicle to be found in the thymus of 16- to 19-day embryos will be described later (p. 256).

Part III: 16½- to 20-day embryos. In embryos of 16½ days (fig. 6) the thymus as a whole is larger, with a flattened base

resting on the pericardium and extending further ventrally than the rest of the organ. Cranially and dorsally each lobe tapers to an ill-defined head lying at the level of the lower third of the lateral thyroid lobe and slightly caudal to the parathyroid, from which it is now separated in all but a few cases. This head may sometimes occupy an abnormally high position (see p. 256). Not only is each lobe subdivided by wide connective tissue septa, but multiplication of epithelial cells and ingrowths of mesenchyme have resulted in the formation of many small bud-like lobules, giving the surface of the thymus a scalloped appearance. In addition to the former plexus of small blood vessels supplying the thymus there now appear in many cases a definitive artery and vein, entering each lobe laterally about one-third of the distance from base to apex. The artery arises from a small branch of the carotid which runs ventrally into the thorax and appears to represent the future internal mammary artery. It is accompanied for a considerable distance by a vein which empties into the jugular. More direct venous connections from thymus to jugular may be present. Inside the thymus the main branches of the vessels tend to run cranially and caudally in the longitudinal septa.

The cytological picture presented by the thymus at this time is somewhat different from any earlier one. Lymphocytes, chiefly large-sized, are far more numerous than before and are scattered throughout the thymus, although more tend to lie in the periphery of the lobules than in their centers. Large lymphocytes are also very abundant in the mesenchyme. While some vacuolated epithelial cells may be found in every part of the thymus, there now appears a peripheral zone where vacuolation is increasing to a considerable extent, resulting eventually in the formation of an apparently syncytial reticulum. Many lymphocytes occupy the spaces of the reticulum but are not sufficiently numerous to obscure them. This peripheral zone, an early stage of which appears in figure 7, will later become very conspicuous along the lateral margins of the thymus (see p. 256 and figs. 8 and 10); the median surfaces of

the lobes, in close apposition, do not usually exhibit as much reticulation. The marginal vacuolation may sometimes extend to the extreme outer limit of the thymus but generally each of the bud-like lobules is covered by a cap of non-vacuolated epithelial cells; figures 10 and 16, showing portions of the peripheral zone from an older embryo, illustrate this condition. At 16½ days, also, the earliest medullary areas appear (figs. 6, 7) as islands or strips of hypertrophied epithelial cells whose nuclei have enlarged and lost much of their staining capacity and whose cytoplasm has become more eosinophilic. These areas tend to run axially throughout the thymus and extend as a core into many of the lobules. In them mitoses are rare and few lymphocytes can be seen; small groups of dark-staining granules may occasionally be present.

In 17-day embryos no great changes are apparent. The thymus lobes are larger and more compact, with much-lobulated edges. The lateral artery and vein are always present in this and all later stages. While the medullary areas have increased but little, the peripheral zone is wider and more prominent along the lateral margin of each lobe. The numerous lymphocytes, about half of which are now of the small variety, are concentrated in this zone, but so wide are the spaces of the reticulum that the region still looks more or less empty.

By 17½ days the thymus lobes are still broader and the septa between the small surface lobules are deeper. Medullary areas are more prominent and in them a few nuclei tend to be arranged in a concentric manner faintly resembling a Hassall's corpuscle. The marginal reticulum forms a still wider lateral zone. Of the many lymphocytes present the percentage of small forms has definitely increased. In embryos of this and all subsequent stages eosinophiles with round or ring-shaped nuclei and conspicuous red granules may be seen lying in the wide, loose septa of the thymus, occasionally also in the surrounding mesenchyme.

The shape and size of the thymus at 18 days is shown in figure 8. There are fewer distinct lobules and the septa are narrower. Medullary areas are variable—in only a few indi-

viduals are they much larger than in the preceding stage. At this time the marginal zone reaches its greatest extent, forming a prominent band along the lateral edge of each lobe (figs. 8, 10). The cytoplasmic strands are now thin and thread-like, with crowds of small lymphocytes between them, although the latter are still not sufficiently numerous to obscure the spaces of the reticulum.

In the few embryos available for the period from 18 days until birth the following gradual changes may be noted: increase in size of the thymus lobes and lobules and in the number of small blood vessels (fig. 12); a relatively very slight enlargement of medullary areas; a gradual fading-out of the marginal zone as its meshes become filled and masked by an increasing population of small lymphocytes.

At this point mention should be made of the vesicles which occur fairly constantly in one or both lobes of the thymus in embryos of 16 to 19 days. These are duct-like structures of clear-cut columnar or cuboidal cells (figs. 6, 8, 9) and may be round, flattened, or triangular in shape. In early stages many are elongated in a cranio-caudal direction (fig. 6) but by 18 days the majority are definitely oriented medio-laterally (fig. 9). Similar structures, which he termed "aberrant vesicles," were described by Rogers ('29) in embryos of 17 and 17½ days; he came to no conclusion regarding their significance. Crisan ('35) believed that vesicles of cuboidal or flattened epithelium observed by him in the cranial part of the thymus of the mouse were caused by degenerative phenomena.

It is possible that these vesicles in the rat represent traces of the ductus pharyngobranchialis or the residual lumen of pouch III; more probable is a relation to the thymic cord. They are first noticeable at 16 days when the cord is beginning to rupture and in a few cases are located at the point where the cord attaches to the thymus. Very frequently, however, the vesicles lie far dorsal or lateral to this point, and their medio-lateral elongation seems quite unrelated to any traction associated with the cord. In a few individuals in which the head of the thymus occupies an uncommonly high position—at

or even cranial to the level of the thyroid and parathyroid, and joined to the body of the thymus by a narrow neck—a vesicle lies at the junction of head and neck (fig. 11), while the thymic cord also attaches to the neck at a point which may or may not coincide with the vesicle. While no positive conclusion can be drawn from such conflicting evidence, it would seem that the structures in question are either related to the thymic cord in some way or are new developments in the thymus due to local growth conditions not otherwise apparent.

Part IV: postnatal thymus. The following brief account is based on sections of thymus secured from only a few animals at 2 hours and at 6 hours after birth and is by no means a comprehensive study of this period.

The thymus has become much larger and more elongate (fig. 13), with narrower septa and somewhat angular lobules, each clearly differentiated into cortex and medulla. Figures 13 and 14 show the greatly increased extent of the medulla at this time. Among its epithelial cells a few small lymphocytes may be found. In the writer's opinion there are no medullary structures sufficiently differentiated to warrant the term of Hassall's corpuscles. The cortex consists of a dense mass of small lymphocytes, with an occasional large, pale nucleus as the only indication of the original epithelial groundwork. It is possible in some cases to distinguish a somewhat less dense peripheral region (fig. 14) which recalls the marginal zone of earlier stages. Small lymphocytes are very numerous in the connective tissue septa, often lined up in rows; if, as seems probable, these are migrating away from the thymus, they must remain distributed in the connective tissue or enter the lymph vessels, since very few can be seen in the veins. No vesicles are present in this material but as the entire thymus was not serially sectioned in each case there is no proof that these may not occur. The medulla sometimes contains several small cysts walled only by the irregular epithelial cells; these do not resemble any of the vesicles previously described.

Very noticeable in and around the postnatal thymus are eosinophiles and mast cells. The former are more numerous

than before in the connective tissue, often occurring in groups; a few may now be found within the cortex and medulla of the thymus. Mast cells, appearing for the first time at this stage, are larger than the eosinophiles and are filled with fine blue granules. They too lie in the connective tissue, generally very near a blood vessel. On the basis of nuclear resemblances and transition stages both eosinophiles and mast cells are judged to be differentiating from a lymphocytic cell type as is seen to occur so frequently in the connective tissue of the rat.

DISCUSSION

The thymus of the rat develops from the ventral diverticulum of pouch III. Its general growth, lobulation, and vascularization present no unusual features and have already received sufficient mention. The development of the epithelial and mesenchymal components of the organ may be summarized as follows:

The activity of the mesenchymal cells may be divided into two overlapping periods—early lymphocyte immigration (14–16½ days), and, after 16 days, extensive multiplication of these cells within the thymus and subsequent emigration. As early as 14 days a few large lymphocytes are to be found lying in clear spaces among the epithelial cells at a time when no connective tissue septa or blood vessels have yet entered the thymus. The characteristic nuclear and cytoplasmic staining of these cells as distinct from the epithelial elements and the appearance of immigration stages at the edge of the thymus make it evident that they are true lymphocytes from the mesenchyme. In later stages many more lymphocytes enter the thymus, especially from the connective tissue septa; however, an increase in number through immigration goes on much more gradually in the rat than in many other mammals. This may be correlated with the relative scarcity of free cells in the surrounding mesenchyme in early stages. After 16½ days immigration apparently declines and the number of lymphocytes within the thymus is increased by mitotic division, with a resulting high percentage of small forms. By 18 days groups

of small lymphocytes in the septa appear to be migrating away from the thymus, although it is not always possible to determine whether cells are entering or leaving the organ. Similar processes of immigration, multiplication, and emigration were described by Maximow ('09).

In the cell-rich mesenchyme surrounding the thymus free cells are few until about 16 days, when for a time they become numerous, the majority being large and medium-sized lymphocytes. Erythrocytes in the blood vessels resemble these cells but contain hemoglobin. No foci of erythropoiesis such as found by Maximow ('09) in the mesenchyme of a 19-mm. rat and by Badertscher ('15) in the thymus septa of pig embryos occur in this material. Eosinophiles are found singly in the thymus septa after $17\frac{1}{2}$ days and are more numerous after birth. Similar cells were described by Maximow ('09) in rats of the same stage of development, by Weill ('13) in postnatal rats, and by Badertscher ('20) in postnatal pigs. Mast cells are found in this material only in the postnatal thymus, although reported by Maximow ('09) in 19-mm. rat embryos (about $17\frac{1}{2}$ –18 days).

Simultaneously with the mesenchymal changes, the ventral diverticulum from pouch III develops into the characteristic epithelial groundwork of the thymus. In 14-day rat embryos vacuoles appear in certain of the epithelial cells and lymphocytes entering at this time come to lie in spaces already formed. During the growth of the thymus increasing vacuolation results in the formation of an extensive reticulum with epithelial nuclei widely separated and cytoplasm reduced to fine strands. Very conspicuous in 17- to 18-day stages is the marginal zone previously described where the lymphocytes are not sufficiently numerous to obscure the wide spaces of the reticulum. With expansion of the medulla and multiplication of small lymphocytes this region disappears, although traces of it may be seen in some sections of postnatal thymus. The question arises as to whether the formation of the reticulum is a primary reaction of the epithelium or is secondarily induced by the invasion of lymphocytes. In several mammals (cat, guinea pig,

rabbit), as reported by Maximow ('09) and Badertscher ('15), the reticulum appears to be secondary and does not become extensive until after lymphocytes are present in quantity. In the rat, however, the reticulum must be considered to be primary, since the small number of lymphocytes present up to 16 days is entirely insufficient to cause so great a vacuolation, at least from mechanical reasons. This was noted by Maximow ('09), who particularly mentioned the rat as favorable material for the study of the reticulum since in embryos up to 20–25 mm. (18–19 days) its meshes are not concealed by lymphocytes.

Medullary areas can be distinguished at 16½ days as islands or strips of hypertrophied epithelial cells with altered staining reactions, forming a core for many of the thymus lobules. These areas increase very slowly until birth when there is a sudden expansion, apparently causing lymphocytes to be pushed into the cortex. A few groups of concentrically arranged nuclei may occur in the medulla but seem scarcely to warrant being termed Hassall's corpuscles, although Maximow ('09) reported these structures in 19-mm. rats (about 18 days). It is evident that up to the time of birth the rat thymus shows a rather poorly-defined cortex and medulla, while in postnatal material the two parts are more clearly marked. This rapid expansion of the medulla should be followed in stages covering the period immediately prior to and subsequent to birth—material which was not at hand for the present study.

That the entire epithelial reticulum of the rat thymus is of entodermal origin seems clearly indicated. The cervical vesicle can be identified as an entity for a considerable period (until 16 days), after which time it may be present in a degenerating condition. No indication can be seen of any migration of cells from it to the thymus, as described by Norris ('38) for man. To be sure, the rat possesses an epithelial arrangement of the superficial reticulum material, with bud-like, scalloped lobules similar to those mentioned by Norris, but the caps of cells on these lobules are quite clearly non-vacuolated epithelial elements of the original thymus anlage and show no correspond-

ence to those of the cervical vesicle. Badertscher ('15) in the pig and Maximow ('09) in guinea pig, rabbit, cat, and rat, also observed a peripheral zone of unaltered epithelium around the thymus at a certain period. The writer feels convinced that the lobulation is solely the result of continued growth of the original epithelial cells from the pharyngeal pouch, with no ectodermal contribution.

Very striking throughout development is the localization in the thymus alone of epithelial vacuolation and lymphocyte immigration. Neither process is evident in the other pharyngeal derivatives. Whether some common factor causes both reactions to occur, or whether such a factor is responsible only for the initial vacuolation and this in turn attracts the lymphocytes, cannot be determined.

SUMMARY

In rat embryos during the thirteenth day of development the thymus anlage (ventral diverticulum of the third pharyngeal pouch) is entirely epithelial and cannot be seen to differ cytologically from the other pharyngeal derivatives. From this time through the sixteenth day there occur: growth and caudal displacement of the thymus, with separation of the parathyroid from it and invasion of both organs by connective tissue and blood vessels; increasing vacuolation of the epithelial cells of the thymus to form a primary reticulum; slow immigration of large lymphocytes into the thymus from the surrounding mesenchyme, beginning in advance of any connective tissue ingrowth. Between 16 days and the time of birth immigration decreases, and multiplication of small lymphocytes takes place in the meshes of the thymus reticulum. During this period small medullary areas appear and a conspicuous marginal zone of the reticulum develops along the lateral and ventral edges of the thymus. The thymus shortly after birth consists of a greatly expanded medulla and a cortex crowded with small lymphocytes. Well-defined Hassall's corpuscles are not present.

The ectodermal cervical vesicle can be identified as a constant structure until 16 days, after which time it is variably present in a degenerating condition. It appears to make no contribution to thymus epithelium, which is therefore purely entodermal in origin. Other, duct-like vesicles in the cranial pole of the thymus appear to be related in some way to the thymic cord.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

- 1 Dorsal diverticulum of pouch III, 13½-day rat embryo; lateral to it, the hollow cervical vesicle, touching ganglion nodosum. Ductus branchialis III at left. $\times 50$.
- 2 Complex III, 14-day rat embryo, showing dorso-medial parathyroid anlage (deeply stained), thymus extending caudally between aortic arch IV and nerve XII, cervical vesicle related to thymus and ganglion nodosum. Remnant of pharyngobranchial duct medial to parathyroid. $\times 50$.
- 3 Complex III, 14½-day rat embryo. Partial separation of parathyroid and thymus, caudal extension and lobulation of thymus, cervical vesicle dorso-lateral to thymus, lying against nerve XII. $\times 50$.
- 4 Thymus, parathyroids, thyroid isthmus in 15-day rat embryo. Cervical vesicle present on right side. $\times 50$.
- 5 Thymus, parathyroids, lateral thyroid lobes of 16-day rat embryo. Cervical vesicle and thymic cord present on left side. $\times 50$.

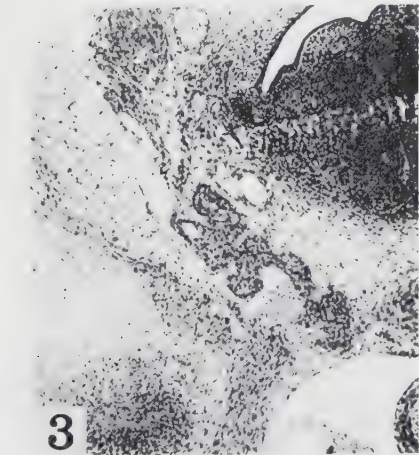
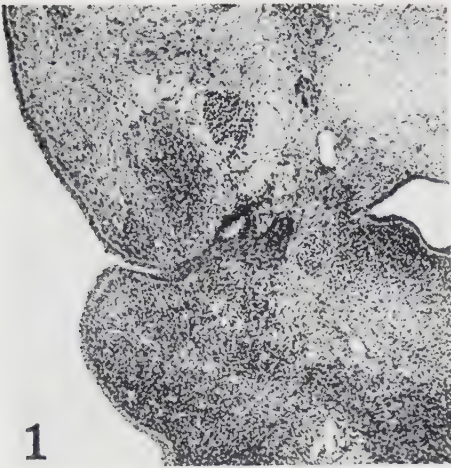


PLATE 2

EXPLANATION OF FIGURES

6 Thymus, 16½-day rat embryo, showing surface lobulation, early medullary areas, vesicle located in cranial pole of left lobe. $\times 50$.

7 Portion of figure 6 under higher magnification. In pale-staining medullary area epithelial nuclei are enlarged. Spaces between peripheral epithelial cells indicate beginning of marginal zone of reticulum. Dark nuclei are those of lymphocytes. $\times 330$.

8 Thymus, 18 day rat embryo. Marginal zone appears as light border along lateral side of each lobe. Small artery enters right lobe laterally. $\times 50$.

9 Portion of figure 8 under higher magnification to show elongate vesicle in cranial pole of left lobe. Vacuolated cervical vesicle lies dorso-lateral in the connective tissue. $\times 192$.

10 Portion of figure 8 under higher magnification, showing marginal zone of reticulum. Small dark nuclei are those of lymphocytes. Toward upper right, cap-like arrangement of peripheral epithelial cells. $\times 192$.

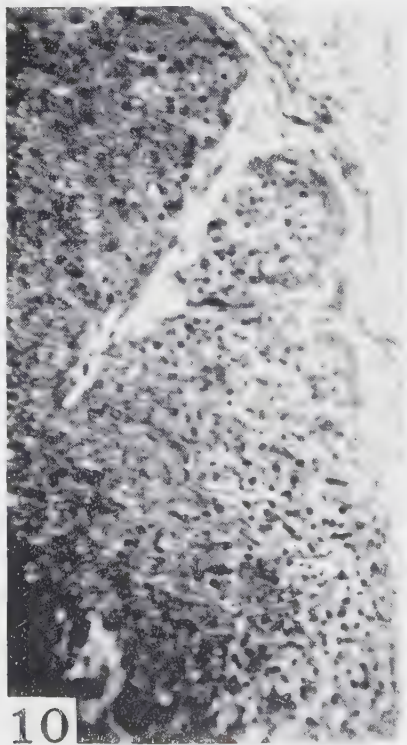
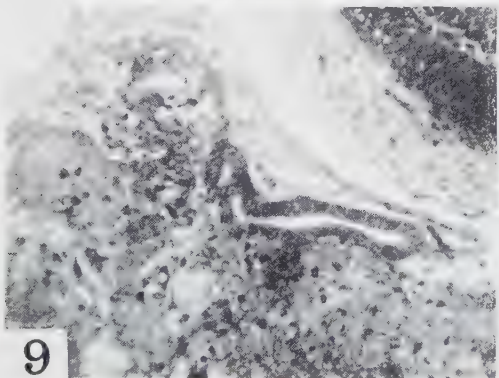
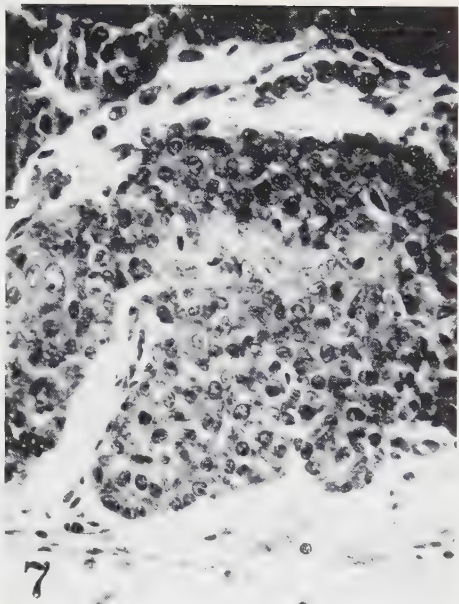
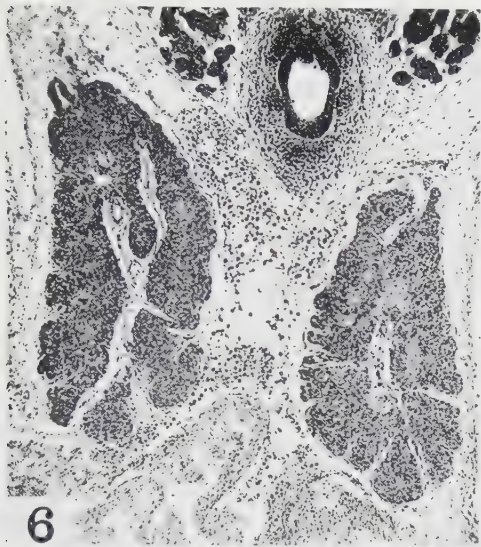


PLATE 3

EXPLANATION OF FIGURES

11 Thymus, thyroid, parathyroid, 17-day rat embryo. Persistent thymic cord extends from parathyroid to thymus neck, where a vesicle is located. $\times 50$.

12 Thymus, 19-day rat embryo. Numerous white spots are small connective tissue septa bearing blood vessels. $\times 30$.

13 Thymus of rat 6 hours after birth, showing greatly expanded medulla and dark-stained cortical areas. $\times 30$.

14 Thymus lobules of rat 6 hours after birth. Peripheral region of less density represents former marginal zone of reticulum. $\times 65$.

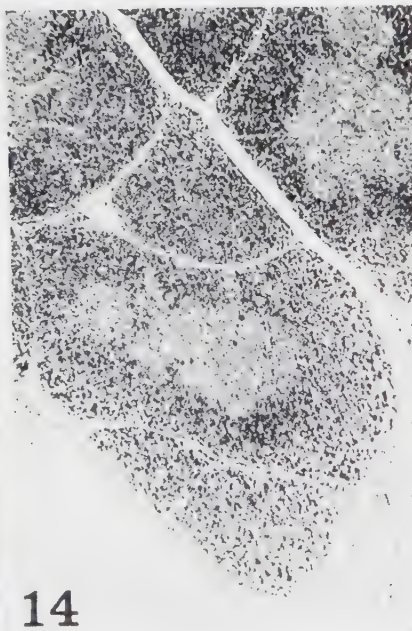
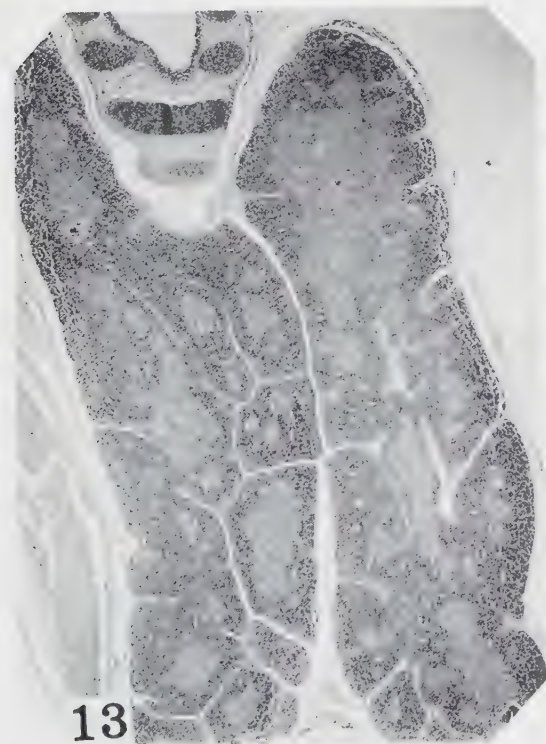
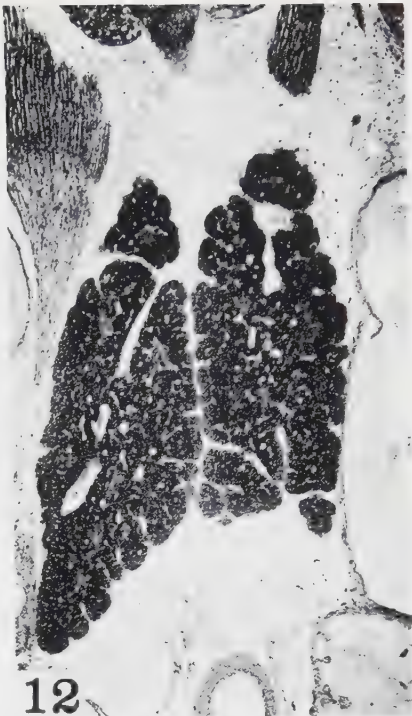
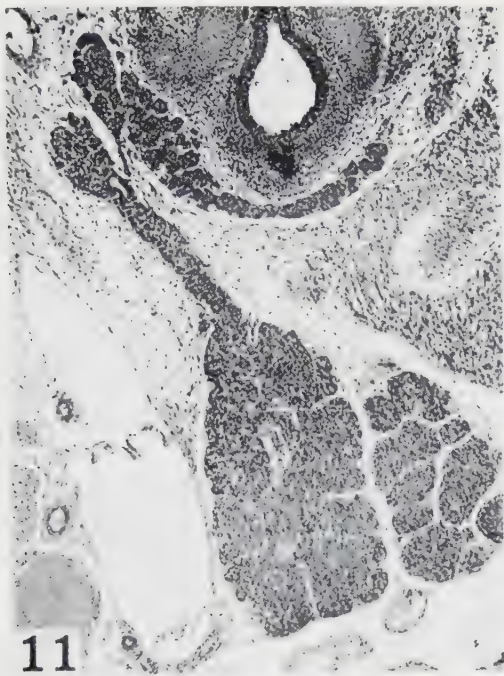


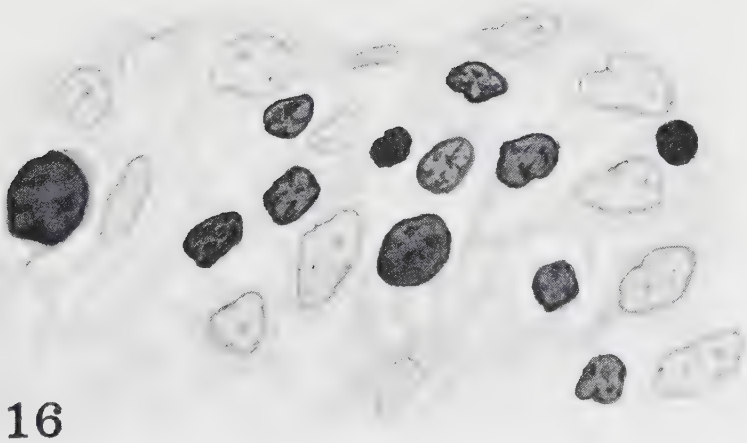
PLATE 4

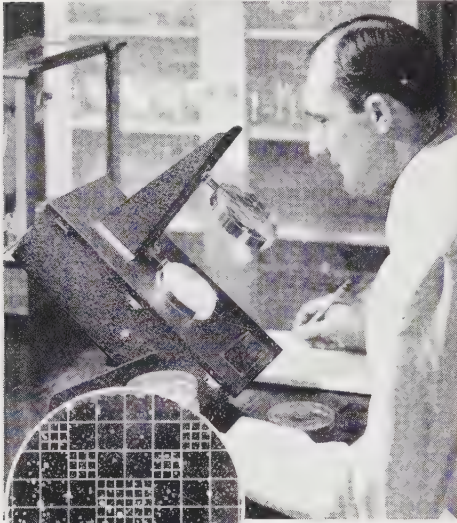
EXPLANATION OF FIGURES

15 Portion of thymus, 14-day rat embryo, showing two large lymphocytes among epithelial cells which are in an early stage of vacuolation.

16 Portion of marginal zone of thymus, 18-day rat embryo, showing epithelial reticulum, large and small lymphocytes (dark-stained), cap-like arrangement of peripheral epithelial cells.

(Figures 15 and 16 were sketched by projection microscope at a magnification of $\times 2000$.)





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MINERAL DISTRIBUTION IN THE DEVELOPING TOOTH¹

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the School of Dentistry, St. Louis, Missouri*

THREE PLATES (EIGHTEEN FIGURES)

INTRODUCTION

There exists little, if any, precise information about mineral distribution during tooth development. Perhaps the technical difficulties involved in securing such data have deterred investigators. This study was undertaken with the idea of supplying some of the details in this gap in our knowledge of tooth structure. The microincineration method offers a means of accurately localizing the total heat resistant minerals and is applicable to developing dental tissues. It is not unlikely that the data obtained by this technique can, in the future, be correlated with that secured by the use of soft x-rays (Applebaum, '38) the polarizing microscope (Kitchin, '33) and artificially radio-active salts so that a connected and orderly history of the minerals in odontogenesis may be evolved.

The methods used in this investigation are primarily those of Scott ('33, '37) and have been described by him in detail. Only the briefest summary of them will be given here. Small pieces of tissue were fixed in a mixture of absolute alcohol and 10% neutral formalin, dehydrated by passage through

¹ This work was supported by a grant from the Carnegie Corporation of New York.

² The material presented is taken from a thesis submitted in partial fulfillment of the requirements of Washington University for the degree of master of science.

³ The author wishes to express his appreciation and thanks to Dr. Gordon H. Scott for the suggestion of the problem and for helpful criticism during the course of the study.

a number of changes of absolute alcohol, cleared in xylol and embedded in paraffin in the usual manner. Sections 4 to 5 μ thick were placed on glass slides and flattened by gentle pressure. These preparations were incinerated according to the method established in this laboratory. While the slides were still warm and therefore unable to condense moisture from the air, a cover glass was sealed over the preparation by means of a beeswax and paraffin mixture.

The heat resistant minerals remain, as far as can be demonstrated, in the same topographic relationship as in the fixed tissue. With no other technical procedure available it is possible to secure a picture of the total mineral distribution in biological tissues. Microincineration offers the other advantage that some of the elements making up the total can be recognized by the color of their oxides and carbonates. For example, iron is easily detected by the yellow to red brown color of its oxide. The depth of color is apparently roughly proportional to the amount of iron involved. This finding has been checked in many tissues by means of the quartz spectrograph. Calcium and magnesium carbonates give a characteristic highly refractive white ash which with a little experience can be consistently recognized. In striated muscle this ash has been clearly demonstrated to be calcium and magnesium by the electron microscope in the hands of Scott and Packer ('39 a and b).

The tissues used in this study were obtained from embryos of cats, dogs, guinea pigs and man; and these were supplemented, whenever available by human material. The tissues used were not confined to animals of one litter but were taken from a large series. Naturally this procedure tends to smooth out individual differences in favor of the observations of apparently general applicability. The variations in isolated animals or in those of similar genetic or nutritional history are doubtless highly significant but were considered outside the scope of this investigation as they merit detailed study and experimental treatment.

OBSERVATIONS AND COMMENT

Oral epithelium. Since the enamel organ arises from oral epithelium a study was made of this tissue with the hope that early evidences of chemical differentiation might be uncovered. Figure 1 represents a photomicrograph of an incinerated section of oral epithelium from a 28-week human embryo. The residue of the cuboidal cells of the malpighian layer is conspicuous and is made up of the very diffuse white refractile ash suggestive of a high percentage of calcium and magnesium. The ash of the outermost cells of the epidermis show a decided diminution of the Ca and Mg ash and are relatively mineral free.

By far the greatest part of the white ash is left by the incinerated nuclei of the epithelial cells and a close comparison with control stained sections indicates that the chromatin material is in all probability its source. The particles of mineral vary from extremely fine granules to coarse aggregations. The ash residue of the nucleolus is especially conspicuous. Most of the individual mineral beads are flecked with brownish red a color indicative of the presence of iron. This appearance is especially noticeable in the malpighian layer. There are, however, a few nuclei which show little or no iron in their mineral residuum.

Individual cell outlines are easily made out. The cell membrane in the fixed tissue has in it a sufficient amount of mineral to leave a distinct and unmistakable residue which in all probability has a large Ca content. The cytoplasm is represented by a very fine evenly dispersed mineral deposit. Some of this is of the whitish color associated with calcium the remainder is slightly bluish in tinge. This color difference may well have two meanings. It is known that a certain order of separation in the dispersion of particulate material gives rise to a bluish color in the transmitted light. The other possibility is a real chemical difference in the residue. If the latter is the case, the most probable causal agent is potassium as sodium is said to be present in cells in a very diminished amount.

The mesoderm beneath the epithelium leaves a mineral residue closely resembling that of adult connective tissue. It is characterized by outstanding nuclear deposits rather widely separated. These are made up most of the whitish ash of calcium and magnesium. The iron in the nucleus, as evidenced by the brown to red coloring, is much less than that of the epithelial nuclei immediately adjacent. The nucleolar ash is prominent. The cytoplasmic processes of the mesodermal cells yield a rather heavy calcium ash. Tissue spaces and tissue fluid leave little mineral after incineration. What is present is finely dispersed and bluish in color. Sodium may well be responsible for this appearance. The alternative explanation is the degree of dispersion and the size of the particles of the residue.

Dental lamina. During the fifth or sixth week of human embryonic life, the primitive epithelium of the mouth proliferates into the underlying mesenchymal tissue and forms a band that extends around the margin of the developing jaw. This dental lamina is the anlage of the ectodermal portion of the teeth. It is separated from the mesenchyme by a cuboidal or low columnar epithelium with a distinct basement membrane formed by the invagination of the malpighian layer of the oral epithelium. The central area of this structure is composed of undifferentiated epithelial cells corresponding to the stratum germinativum of the epidermis.

Figure 2 is a photomicrograph of a control section, stained with haematoxylin and eosin, of the dental lamina of a guinea pig embryo. The cytoplasm of the cells is colored a light bluish pink but in some cases clear and structureless, particularly in the upper portions of the basal cuboidal epithelium. The cell outlines are quite distinct. The nuclei stain a deep blue peripherally whereas, the central portions show a light bluish tinged network. The nucleoli are easily distinguished.

As might be surmised, the incinerated dental lamina (fig. 4) bears a close resemblance to the oral epithelium from which it is derived. The cells are more or less clearly outlined in fine stippled bright white ash. The cytoplasmic residue of

the basal cells is composed of finely dispersed flat white ash. However, the uppermost portion of the cuboidal cells that did not stain in the control section is relatively free of any mineral deposits. Around the periphery of the nuclei dense deposits of bright white ash are seen, occasionally tinged by reddish brown flecks. As the more superficial strata of cells are approached the flat white cytoplasmic ash deposits become more scarce and assume a bluish hue apparently more intense than that of the oral epithelium from which the lamina is derived. Even the nuclei share in this transition and become less prominent by the loss of large portions of their calcium and magnesium.

Enamel organ. At twenty points, corresponding to the positions of the future deciduous teeth, the cells of the dental lamina proliferate still deeper into the underlying mesoderm. According to Orban ('28) and others this is not a growth downward as usually assumed but is a centrifugal growth with the surrounding tissue.

The developing enamel organ passes through several stages. It appears first as a thickening of cells which assume a bud-like shape, later the basal portion invaginates and grows around the mesodermal structures that will form the dental papilla. At this stage the epithelial bud has differentiated into the mature enamel organ and is still connected to the oral epithelium by a strand of cells, a remnant of the dental lamina.

Figures 5 and 6 are photomicrographs of a control and an incinerated section, respectively, from an early stage in the development of a mandibular first incisor of a guinea pig. It is a rapidly proliferating tooth bud and consequently there are numerous undifferentiated cellular elements. There is a great increase in the ash of calcium and magnesium compared with the oral epithelium and dental lamina previously discussed, especially in the cells around the periphery of the bud. Most of this ash is found to be present in the nucleus and occupies a position similar to the chromatin material in the control section. There are also areas corresponding to

the nucleoli that show very brilliant white globules of ash. An interesting feature of the nucleus is the apparent increase in the amount of iron compared with that in the oral epithelium from which the organ is derived. It seems as though not one nucleus is devoid of this material although some nuclei contain greater quantities than others. Most of this yellow to red mineral residue is found in the periphery of the nuclei. The outlines of the individual cells are difficult to detect. The cytoplasmic deposit is rather scanty and consists of a fine grayish white ash overshadowed by a bluish sheen.

The ash of the surrounding mesodermal cells is less conspicuous due to the reduced number of cells as compared with the developing tooth bud. The tissue spaces contain small amounts of grayish white ash and some bluish salt deposits. The nucleoli stand out as prominent elements of the nuclei. The amount of iron present within the nucleus is much less than that in the neighboring epithelial structures. The cytoplasm leaves a white residue that is also present in the fine protoplasmic extensions of the cells. In the area of the future dental papilla, the connective tissue cells destined to undergo differentiation into dentin and pulp elements exhibit the beginning of a light bluish residue that is found both intracellularly and in the tissue spaces.

Figure 7 is a photomicrograph of a control section of a still further advanced stage of development in which the enamel organ is pear-shaped. The cells around the periphery of the organ are relatively undifferentiated elements derived from the malpighian layer of the oral epithelium. They vary from low cuboidal cells in the area that will become the outer ameloblastic epithelium, to tall columnar cells at the base of the organ which will later form the ameloblasts. The central portion of the organ consists of round and polyhedral cells of the stellate reticulum. Numerous mitotic figures are found throughout the organ.

The corresponding incinerated section is shown in figure 8. The outermost layer of cells surrounding the organ is definitely

separated from the surrounding tissue by virtue of its content of white mineral residue for the most part due to the increased number of nuclei. However, when one compares this area with the corresponding one in the preceding photograph (fig. 7), it will be found that the nuclei are not clearly defined due to a decrease in the nuclear ash and an increase in the cytoplasmic residue. This is especially pronounced at the base of the tooth bud. The amount of iron present within the nuclei is little altered. Occasional flecks of brownish red ash are found in the cytoplasmic remnants. The round and polyhedral cells of the central portion of the organ are more widely separated, resulting in a smaller amount of mineral in any given area. The individual cell membranes do not leave a definite deposit of mineral but the cytoplasm is represented by a delicate white ash. The nuclei are discernible but appear to contain less calcium than the corresponding elements shown in figure 6.

Enamel knot, enamel cord and enamel navel. The enamel knot (Ahrens, '13) is a condensation of concentrically arranged cells in the concavity of the enamel organ (figs. 9 and 10). Although its function in amelogenesis is questionable, it has been described as a germinal center for the rapidly growing enamel organ. However, there are apparently no more mitotic figures here than in any other portion of the enamel organ. In the incinerated section the enamel knot is easily recognized by the concentration of calcium salts in comparison with other portions of the organ, due to the increased number of closely packed cells. Most of the mineral is found to be present in the nuclei and consists chiefly of calcium and magnesium. Nucleoli can be made out as globules of white ash. Cell outlines are indistinct and the cytoplasmic residue consists of fine grayish white ash partially masked by a bluish deposition of mineral.

The enamel cord is centrally located in the enamel organ, extending from the enamel knot to the apex of the organ where it terminates in the enamel navel. This cord of cells is believed by some to maintain the invagination of the basal

portion of the enamel organ. The enamel cord consists of groups of interlacing cells with basophilic protoplasmic extensions. The cell membranes are indistinct, the nuclei are elongated and contain a delicate chromatin network with one or two nucleoli.

In the incinerated section the enamel cord is more conspicuous than the surrounding tissue not because of its greater mineral content but rather because of greater concentration of cellular elements in this area. The minerals of the enamel cord are for the most part calcium and magnesium. The deposits of these oxides are interspersed with particles of an ash having a decided bluish tinge. Deposits of yellow to red ash are found around the margins of the nuclei and, to a lesser extent, in the cytoplasm.

Outer enamel epithelium. The outer enamel epithelium originates from the cuboidal epithelium of the malpighian layer of the oral mucosa. In earlier stages of development the cells that make up this structure are cuboidal in shape but soon pass through a series of changes to become elongated or polygonal with their long axes parallel with the free surface of the enamel organ. The outer epithelium may consist of one or more layers of cells that gradually merge with the stellate reticulum. Although the outer enamel epithelium serves as a limiting membrane its function in amelogenesis is much in debate.

An incinerated section of the outer enamel epithelium (fig. 11), reveals the relative amounts of mineral within the area. The elongated epithelial cells contain ovoid nuclei and are closely packed together. Within the epithelium two rather large capillaries can be seen. The residue of the nuclei of the epithelial cells consists chiefly of the white ash of calcium with occasional brownish red particles that are for the most part confined to the periphery. The nucleoli appear as dense globules of highly refractive mineral. The cell membranes cannot be distinguished. The cytoplasmic ash is scattered and is of the same general nature as that of the nuclei with the addition of large amounts of blue ash.

Stellate reticulum. In the early bud stage of development of the enamel organ the centrally located cells derived from the oral mucosa pass through several stages of differentiation to form the mature stellate cells. These cells are connected by fine protoplasmic processes with round or slightly oval nuclei, containing one or more nucleoli.

The incinerated sections (figs. 6, 8, 10 and 12) show the stellate reticulum in various periods of development. The cellular residue in all stages is that of the white ash associated with the presence of calcium and magnesium. In the earlier stages of development the cells proliferate rapidly and are crowded together so that the density of the ash deposits make it difficult to resolve cellular detail with any degree of accuracy. As the tissue becomes more differentiated the number of cells, as well as the amount of calcium and magnesium present decreases. Simultaneously there is a gradual and definite increase in the bluish ash so that, in the fully developed stellate reticulum, there is an almost continuous sheet of intense bluish residue over the entire background. Figure 12 shows incinerated mature stellate reticulum in which the individual cells with their communicating protoplasmic extensions are well defined. No definite ash deposits are present to indicate the cell membranes. The white cytoplasmic residue is very delicate and of the same general type as in the nuclei where the deposits are very dense. The nucleoli are difficult to distinguish. Scattered within the inter-cellular spaces small globules of brilliant white ash and some extremely fine deposits of material similar to that of the cellular residue are occasionally found.

Stratum intermedium. The stratum intermedium consists of a condensation of cells similar to those of the adjoining stellate reticulum. The bodies have elongated protoplasmic extensions communicating with the processes of the stellate reticulum. The nuclei are slightly elongated or oval and parallel the surface of the ameloblasts.

Figures 13 and 15 represent incinerated sections of the stratum intermedium and related structures. The photo-

graphs demonstrate quite well the manner in which the ash is deposited within the cells. The residue in the nuclei and, to a lesser degree, in the fibrillar protoplasmic processes of the cells, is of the white variety associated with calcium and magnesium. Coincident with this ash there are relatively large quantities of bluish residue which increases in amount and reaches a maximum with the beginning of amelogenesis.

Ameloblastic layer. The internal epithelium or ameloblastic layer is an important structure of the enamel organ. It forms the innermost layer of the organ and prior to the apposition of enamel is in intimate contact with the dental papilla. The ameloblasts are responsible for the secretion of the material that enters into the formation of the enamel. These cells represent the ultimate differentiation of cells derived from the cuboidal epithelium of the malpighian layer of the oral epithelium and have passed through many stages before assuming their mature, tall-columnar form.

Study of the various portions of the developing enamel organ shows that those cells destined to become ameloblasts contain large quantities of the ash of calcium and magnesium and probably considerable phosphorus which caused great difficulty in obtaining completely incinerated sections. As the cells continue to differentiate the amounts of calcium and magnesium, as well as phosphorus, gradually diminish. Simultaneous with the decrease in these elements there is an apparent gradual increase in the bluish residue. The decrease in total mineral residue is continued until just prior to enamel formation when the nuclei are migrating to the distal poles of the cells. In the more differentiated ameloblasts, the cytoplasm is practically free of all calcium and magnesium. However, the cell membrane still leaves a fine white stippled deposit of ash that is concentrated toward the basal pole. This basal piling up of ash corresponds with Williams (1896) internal ameloblastic membrane (see figs. 13, 14 and 15).

Fully differentiated ameloblasts that have just begun to function in the formation of enamel reveal a direct reversal of the foregoing condition. Figure 16 is of such a group of

ameloblasts torn from the apex of the cusp of an enamel organ. The nuclei, which have taken up positions at equivalent levels in the distal portions of the cells, are very rich in calcium and magnesium. Iron can be found in all nuclear deposits and the amount present is apparently greater than in any other stage of development. The nucleoli which are very difficult to make out are probably partially obscured by the other mineral particles.

The cytoplasm is the most interesting feature of the cells and leaves heavy deposits of mineral in the basal and distal thirds of the ameloblasts with practically no ash in the central portions just beneath the nuclei. The mineral residue has a definite bluish hue and is interspersed with a few discrete globules of bright white highly refractive particles. This increase in mineral within the cell bodies is apparently closely associated with the deposition of calcific material in the formation of the enamel.

Dental papilla. Soon after the epithelial enamel forming cells make their appearance, the mesodermal cells begin to condense and accumulate beneath the basal portion of the dental lamina. This mesodermal change is the first evidence of the future dental papilla. The dental papilla enters into the formation of the dentin and pulp which comprise the greater portion of the tooth substance.

Incinerated sections of early stages in development of the enamel organ (fig. 6) show no indication of an increased number of mesodermic cells at the basal portions of the structure. These cells are still widely separated and the intercellular fluid leaves a very small amount of mineral residue. The fiber-like extensions of the cells leave a residue of dense white ash of calcium and magnesium; the nuclear deposits are of the same general variety but considerably larger in extent. Occasional flecks of reddish brown ash are also present. The entire background is covered by a very light bluish mineral that is somewhat more accentuated in the area of the future dental papilla.

Figures 17 and 18 are control and incinerated sections, respectively, of a mature dental papilla. There is a very definite increase in the calcium and magnesium content in both the cytoplasm and the nuclei and at this time the intra- and extra-cellular bluish background-ash is quite intense and at its maximum.

Odontoblasts. Before calcification begins there is no distinct difference in the cells around the periphery and those comprising the bulk of the dental papilla. Within a short time there appear, at the apex and along the margins of the dental papilla, accumulations of small round or polyhedral cells having large round or slightly oval nuclei with a very scanty amount of cytoplasm and very delicate protoplasmic processes. Figure 14 illustrates an incinerated section of such an area from a 28-week-old human embryo. The nuclei contain large quantities of bluish residue interspersed with some particles of white ash concentrated around the peripheries of the nuclei leaving the centers practically clear. The cytoplasm and its processes leave a residue of the same general character and concentration causing some little difficulty in outlining the nuclei with any degree of accuracy.

Shortly thereafter, the small round cells at the circumference of the dental papilla undergo further differentiation into tall cylindrical odontoblastic cells which are arranged in palisades and have cytoplasmic processes. Figures 13, 14 and 15, are photomicrographs of incinerated sections of groups of such cells. Unlike the cytoplasm of the differentiating ameloblasts, the cytoplasm of the odontoblasts apparently has no stage in its differentiation where it is without mineral. It contains large quantities of bluish ash and appears to acquire more white residue as it continues to develop. The dentinal fibrils appear to consist of the same dense accumulation of ash found in the cytoplasmic residue. It is quite difficult to outline the nuclei with any degree of certainty due to the similarity in the quantity and quality of the inorganic residue.

SUMMARY

The constancy of mineral distribution, as revealed by microincineration, in adult tissues has been demonstrated repeatedly. In embryonic tissues the situation is quite the reverse as there is evidence of a "continual shift in quality and quantity for each tissue" (Scott, '37). This process is seen especially well in the developing dental organ. In early stages of tooth development cells destined to become enamel forming elements contain relatively large amounts of calcium and magnesium. During differentiation of the ameloblasts the amount of this particular nuclear and cytoplasmic residue decreases markedly. So great is this diminution that at times the cytoplasm is practically devoid of calcium and magnesium. With the loss of calcium and magnesium there is a gain of other minerals, probably largely potassium. In the fully differentiated ameloblast just prior to the formation of enamel the cytoplasm again reveals large quantities of calcium and magnesium especially in the distal and proximal thirds of the cell. The middle third of the ameloblast remains relatively free of calcium and magnesium.

The mineral fluctuation observed in the differentiation and development of the enamel forming cells is in sharp contrast to conditions found in the odontoblastic layers of the tooth germ. The odontoblasts seem to be remarkably constant in their mineral content at every stage of their development. They contain a distinctly greater amount of calcium and magnesium than do their neighboring cells the mesenchyme of the dental papilla. There is some evidence that the more mature these cells become the greater is their calcium and magnesium content. Perhaps the odontoblast is very early a nearly differentiated cell and that its role in dentin formation is a passive one. It might be expected that any cell group actively engaged in a calcifying process might show at some physiologic stage a variation, possibly even a decrease, in their calcium content. This presupposes that calcification takes place in rhythm and is not a steady accretion for which idea there is a certain amount of evidence.

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PLATE 1

EXPLANATION OF FIGURES

- 1 Incinerated section of oral epithelium of 28 weeks human embryo. $\times 430$.
- 2 Control section of a dental lamina of a guinea pig embryo. $\times 430$.
- 3 An incinerated section of a lip furrow band of a guinea pig embryo. $\times 970$.
- 4 Incinerated section of a dental lamina of a guinea pig embryo. $\times 430$. This figure should be compared with its control section shown in figure 2.
- 5 and 6 Control and incinerated sections from an early stage in the development of a mandibular first incisor of a guinea pig. $\times 430$.

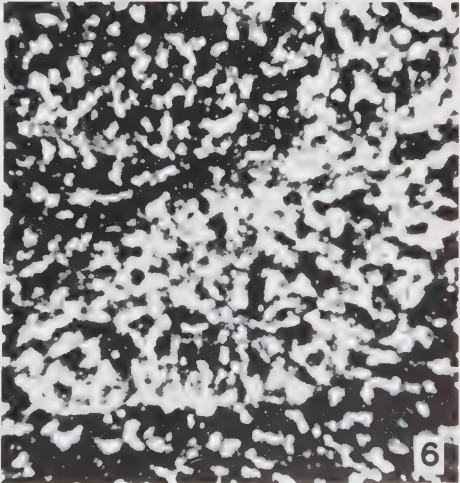
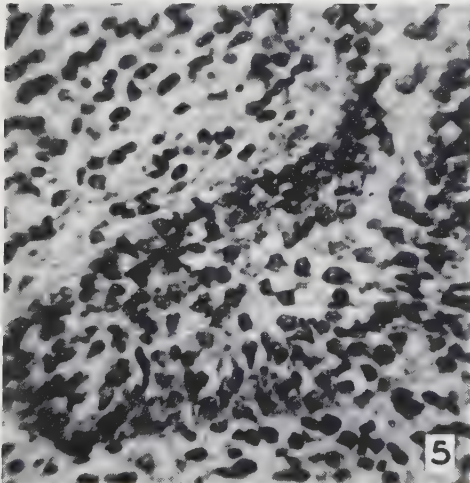
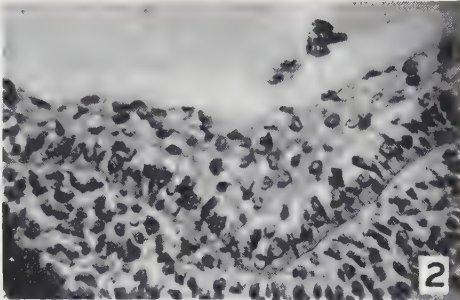
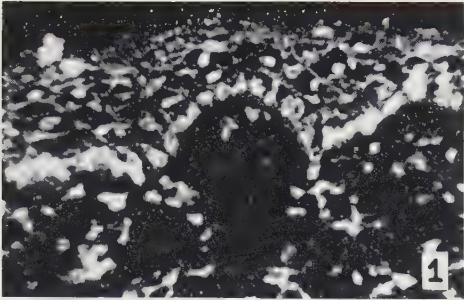


PLATE 2

EXPLANATION OF FIGURES

7 and 8 Control and incinerated sections of a guinea pig embryo showing still further advanced stage of development in which the organ is pear-shaped. $\times 430$.

9 and 10 Control and incinerated sections of a mature enamel organ from a cat embryo. Note inner and outer layer of enamel epithelium inclosing the stellate reticulum. The so-called enamel knot, enamel cord, and enamel navel are shown. $\times 430$.

11 Incinerated section of the outer enamel epithelium of a dog embryo. Note two large capillaries. $\times 430$.

12 Incinerated section of the stellate reticulum, cat embryo. $\times 430$.

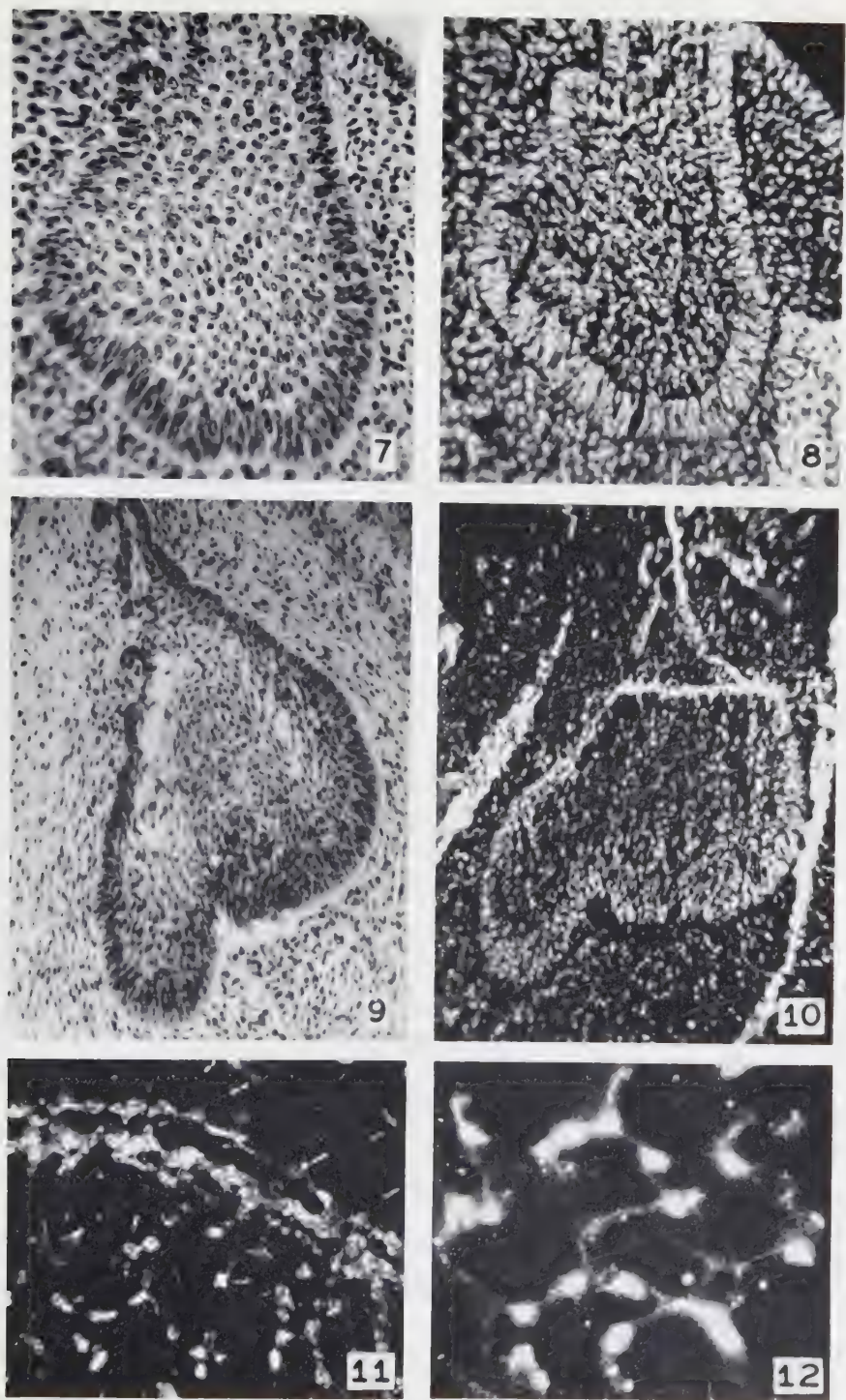


PLATE 3

EXPLANATION OF FIGURES

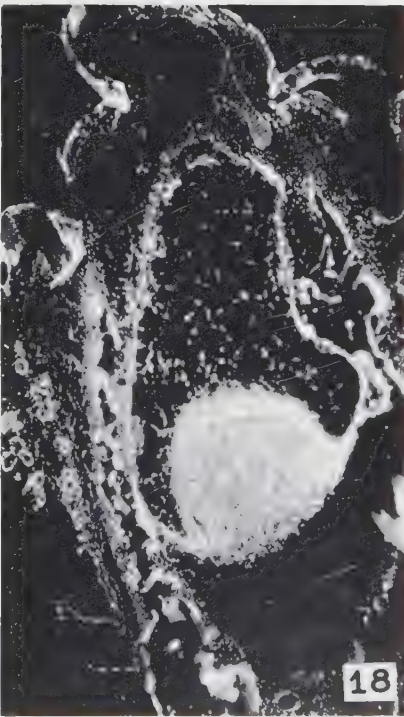
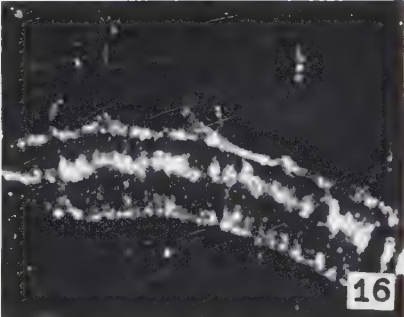
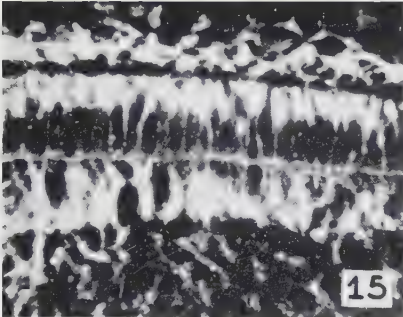
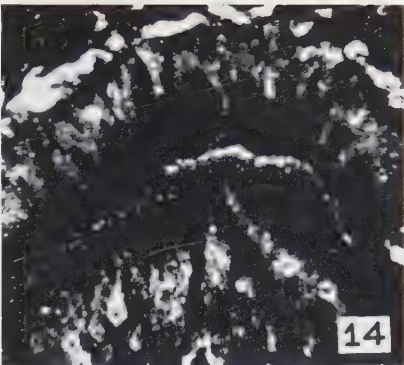
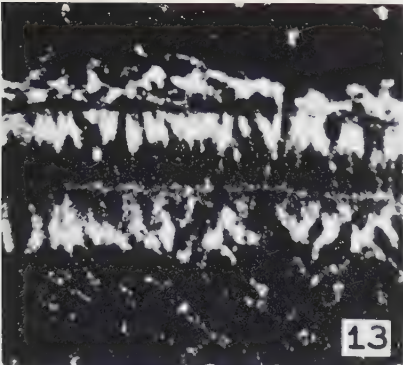
13 Incinerated section illustrating the stratum intermedium, prefunctional ameloblasts and odontoblasts of dog embryo. $\times 430$.

14 Incinerated section showing the stratum intermedium and prefunctional ameloblasts and odontoblasts of a human embryo. $\times 430$.

15 Incinerated section illustrating fibroblasts of the dental pulp, odontoblasts, prefunctional ameloblasts and the stratum intermedium of a dog embryo. $\times 430$.

16 Incinerated section of functioning ameloblasts of a dog embryo. $\times 430$.

17 and 18 Control and incinerated sections of the mature dental organ, showing the enamel and dental forming portions of a cat embryo. $\times 100$.



DEMONSTRATION OF THE NERVE TO THE LEVATOR GLANDULAE THYREOIDEAE MUSCLE

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ONE FIGURE

The nerve which supplies the levator glandulae thyreoideae muscle is encountered rarely. Eisler,¹ who made a careful study of these muscles in 1900, says that "little is known of their innervation." In the absence of reports on the subject subsequent to 1900, Eisler's statement may therefore be regarded as authoritative.

An example of this rare nerve was discovered recently during dissection aimed to determine the exact relationship of the superior laryngeal nerve to the superior thyroid artery.² Of the eighty superior laryngeal nerves thus studied, one possessed an unusual branch on the right side. This branch, upon investigation, proved to innervate the left levator glandulae thyreoideae muscle; it is illustrated in figure 1.

The cadaver was that of a white female, aged 72 years, dead of bronchopneumonia, and was well preserved when dissected. It had been embalmed by injection of 10 liters of 10% formalin into the right femoral artery and into the right common carotid artery. The thyroid gland was normal. No pyramidal lobe was present. Two sets of parathyroid glands were found in their normal locations. Other anomalies noted in this cadaver

¹ Eisler, P. 1900 Anat. Anz., 17: 183-196.

² This investigation was aided by a grant from the Rockefeller Foundation to Washington University for research in science.

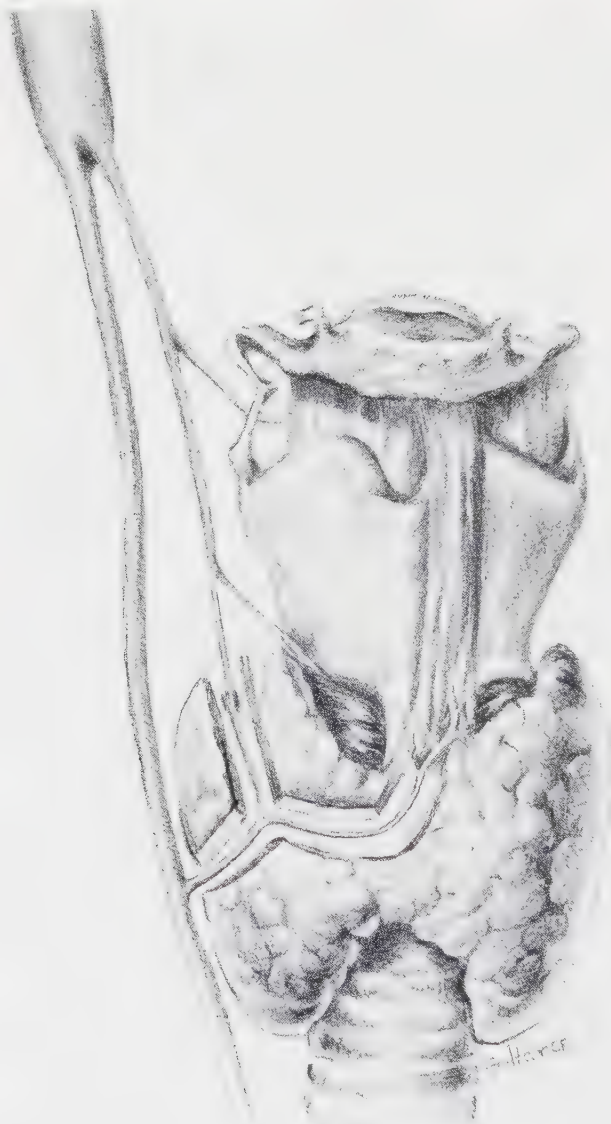


Fig. 1 Showing the origin, course and termination of the nerve branch supplying the left m. levator glandulae thyroideae ($\times 1/1$).

were two extra carpal bones on each side, and an os trigonum in the right ankle.

The nerve originated from the external branch of the right superior laryngeal nerve and terminated in the left levator glandulae thyreoideae muscle. It anastomosed with the right vagus nerve; also came into intimate contact with the right superior parathyreoid gland (gland IV). It ran upon the outer surface of the larynx and trachea covered by the thyreoid gland, coursing first inferiorly, then horizontally to the left beyond the midline, and finally turned superiorly. Below its origin at the level of the lower part of the ala of the thyreoid cartilage, it lay posterior and mesial to the superior pole of the right lobe of the thyreoid gland. Before it anastomosed with the right vagus nerve it came into contact with the right parathyreoid (gland IV). After its anastomosis the nerve lay behind the isthmus of the thyreoid gland, and beyond the midline on the left it coursed upward to enter the muscle on its posterior surface and to ramify within it.

Two levator glandulae thyreoideae muscles were present. The left muscle alone was found to receive a nerve; no nerve was demonstrable innervating the right muscle.

Both muscles were similar in origin and in insertion, and each was symmetrically arranged. They originated from the inferior border of the body of the hyoid bone below the lesser cornu and were inserted into the capsule of the mesial surfaces of the superior poles of the thyreoid lobes. Portions of the nerve and muscle were sectioned and their structure verified microscopically—medullated nerve fibers, striped muscle fibers.

In his article, Eisler describes and illustrates the nerve to the muscle in two cases. In both, the nerve arose from the external branch of the superior laryngeal nerve. One of these nerves arose on the left side and first pierced the pars recta of the cricothyreoid muscle before supplying the left levator of the thyreoid gland. The other had its origin on the right side.

MICROSCOPIC CHANGES IN THE ISLANDS OF LANGERHANS PRODUCED BY SYMPATHETIC AND PARASYMPATHETIC STIMULATION IN THE CAT

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SEVEN FIGURES

In a previous communication (Sergeyeva, '38) relating to the microscopic changes produced by sympathetic and parasympathetic stimulation in the acinous cells of the pancreatic gland of the cat, some changes in the islands of Langerhans were described, but they were not studied in detail. The aim of the present investigation was to determine the relationship between the two sets of autonomic nerves and different cells of the islands of Langerhans. For this purpose additional physiological material was obtained and special histological differential staining methods were applied to the α - and β -cells. Such an investigation seemed the more desirable since apparently no previous workers have studied systematically by combined physiological and histological methods the effect of stimulation or ablation of the nerves to the pancreas on the endocrine tissue of the pancreatic gland.

METHODS

Physiological methods

The experimental animals used in this work were cats and one dog. The methods used in the acute experiments were the same as in the previous work (Sergeyeva, '38).

Prior to the acute experiments in some instances different preliminary operations were performed. In one group of

cats, each animal was operated on twice some weeks before the actual experiment. In the first operation part of the right abdominal sympathetic chain, extending from the pelvic region to the diaphragm, was extirpated, and the right splanchnic nerve was cut below the diaphragm, its peripheral end with the coeliac ganglion then being removed. After the lapse of 10 days or more, the left abdominal sympathetic chain, left splanchnic nerve and left coeliac ganglion were similarly removed. The experiment was performed 3 to 4 weeks after the second operation.

In another group of animals all the vagus branches were previously cut just below the diaphragm or in the chest. In one case the main pancreatic duct was also ligated. The animals were used for acute experiment 4 to 5 weeks after the preliminary operation. In two more cases only ligation of the main pancreatic duct was performed, the animals being killed 2 weeks later.

One cat was fed with cream and sacrificed several hours later.

Histological methods

At the beginning of every experiment control samples (from one to four as occasion permitted) were taken from the pancreas and fixed in different solutions, viz.:

1. Bailey's solution
 - 45 cc. 3% mercuri-chloride
 - 45 cc. 3% potassium bichromate
 - 10 cc. 40% neutral formalin
2. Orth's fluid
 - 100 cc. 2½% potassium bichromate
 - 10 cc. 40% neutral formalin
3. Bensley's solution (acetic, osmic, bichromate—A.O.B.)
 - 8 cc. 2½% potassium bichromate
 - 2 cc. 2% osmic acid
 - 1 dr. glacial acetic acid
4. Bouin's Picro-Formol
 - 30 parts water
 - 10 parts commercial formol
 - 2 parts 2% aqueous solution of trichloroacetic acid
 - Picric acid to saturation

At the end of the experiment the pancreas was fixed by injection of one of these solutions through the aorta, or samples of tissue were fixed by immersion. The tissue was embedded in paraffin, and sections were cut $5\ \mu$ thick. Sections of the tissue which had been fixed in solution 1 or 2 were stained with neutral gentian and Bowie's Biebrich-scarlet, basic ethyl-violet. With sections which had been fixed in solution 3 (A.O.B.) the Mallory-Azan method of staining was used. Some of the material that had been fixed in solution 4 was stained by the method of G. Gomori of The University of Chicago, the remainder by Masson's method of silver impregnation in block.

After fixation of the tissue in solution 1 staining with Bowie's Biebrich-scarlet, basic ethyl-violet gives good differentiation of the α -cells in the islands of Langerhans—the fine granules of these cells staining blue, the β -cells appearing pink and semitransparent. With solution 2, the same dye stains the α -cells pale blue, and certain cells which contain zymogen granules violet in the apical part, the material in the basal part of these cells staining blue like the α -cells. Sometimes the whole cytoplasm of these zymogen-containing cells is stained dull blue and their contours are irregular. These cells may be regarded as acinous cells in the process of transformation into typical α -cells, and may be designated "precursors" of α -cells (fig. 1); they probably constitute a reserve of endocrine α -cells. The "precursors" are situated generally around the islands, but sometimes near the margin of the lobule. They are not very numerous in the normal resting pancreas but are greatly increased in number after certain kinds of stimulation, as described below.

After fixation in solution 3 and Mallory-Azan staining all the epithelial elements in the gland are differentiated: the zymogen granules appear deep red, the granules of the α -cells pink, and the β -cells grey and semitransparent. The centro-acinous cells are similar in morphological appearance and stainable properties to the β -cells.

After fixation in solution 4 and staining with Masson's silver impregnation method the granules of the α -cells appear black or dark brown.

The island cells are not confined to the island areas only. Very small islands, consisting of a few α - or β -cells or of both kinds of cells may be seen in different parts of a lobule. Single α - or β -cells may even be present among the acinous cells, and

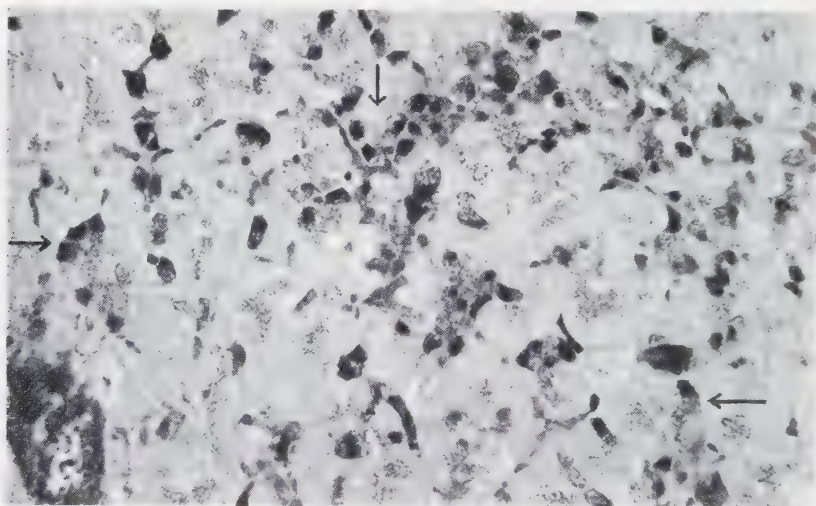


Fig. 1 Showing "precursors" of α -cells. The dark cells, scattered throughout the acinous tissue are "precursors" of α -cells. Some contain dark-stained material (blue in the preparation) at the base of the cell and zymogen at the apical part of the cell (arrows). In some the whole cytoplasm is dark and no zymogen can be seen. The contours of the "precursors" resemble those of α -cells, but some are irregular in shape. Fixation: Orth's fluid. Staining: Bowie's Biebrich-scarlet, basic ethyl-violet. $\times 600$.

these will be called "scattered" α - or β -cells. The stainable properties of these "scattered" α - or β -cells are the same as described for the island cells.

In the normal pancreas there is sometimes seen what may be called provisionally a "transitional" form of cell with clear cytoplasm and definite outlines, containing zymogen granules in the apical part and endocrine granules in the basal part, the latter granules resembling those of the α - or β -cells (fig. 2).

The α -cell transitional forms are more readily and more frequently discernible owing to their more precise differentiation. They are seen sometimes in the normal pancreas but are very numerous after various types of stimulation, e.g. after

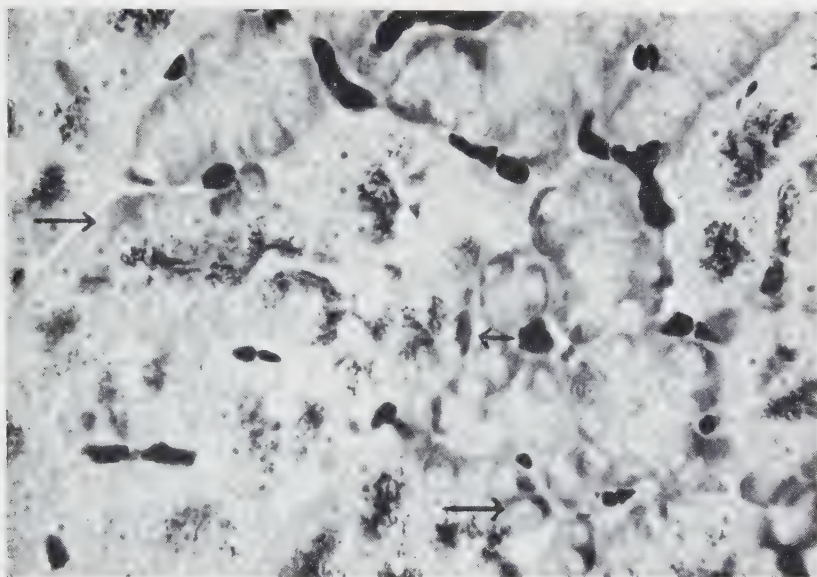


Fig. 2 Showing "transitional" cells. Sample obtained from pancreas of cat 3 hours after feeding with cream. Cell forms transitional between typical island cells and typical acinous cells, representing a more advanced stage in the evolution of the "precursors." The combined cell cords are particularly evident toward the left and lower right of the picture, where the zymogen granules may be seen in the apical part and dark-stained material at the base (blue-stained endocrine material in the preparation) of the cells (arrows). Fixation: Bailey's solution. Staining: Bowie's Biebrich-scarlet, basic ethyl-violet. $\times 600$.

feeding with cream (see fig. 2). They may be regarded as representing a more advanced stage in the evolution of the α -cells than the "precursors."

EXPERIMENTAL RESULTS

Effect of stimulation of the vagus nerves

In three cats the vagus nerves were stimulated rhythmically in the neck with an induction current (coil 14 to 7 cm.). After

several hours' stimulation the following changes could be seen microscopically in the pancreatic tissue. The exocrine cells were depleted of granules. About 60% of the islands were surrounded by "halos" of unexhausted acinous cells. The blood vessels were distended and filled with erythrocytes. The islands had undergone marked changes. The outlines of the cell cords had become indistinct, and vacuoles could sometimes be seen in them. Some of the islands appeared greatly distorted (see fig. 6—Sergeyeva, '38). Many of the cells appeared

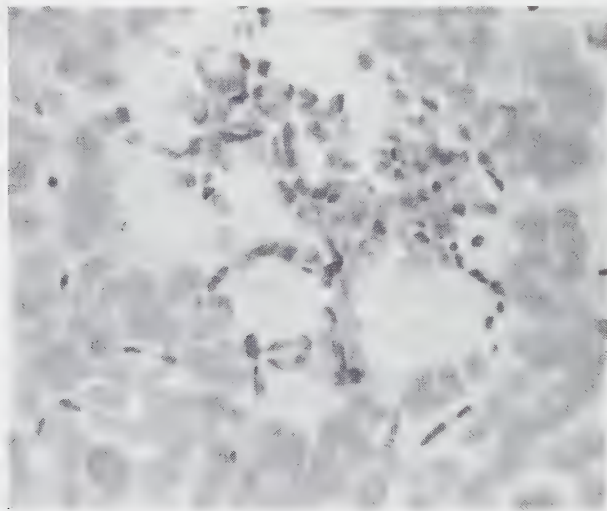


Fig. 3 An island after less prolonged vagal stimulation. The island is increased in size owing to the increase in number of both elements. α -cells are situated in the middle of the island. Fixation: Orth's fluid. Staining: azo-fuchsin ethyl-violet. $\times 320$.

to have a dual nature, both exocrine and endocrine; these were the above-mentioned "transitional" cells.

Less prolonged and intensive stimulation of the vagi caused only a very moderate discharge of zymogen granules. The size of the islands was increased, owing to the appearance of a greater number of β -cells and (though in a lesser degree) of α -cells; the latter tended to remain in the centre of the islands (fig. 3).

In these experiments the blood sugar concentration was determined before and during the experiment, by the method of Hagedorn and Jensen. During the first 2 hours of vagal stimulation the blood sugar concentration rose to a moderate height (minimum 161, maximum 230 mg. per cent); in the next 2 or 3 hours it gradually fell, although never as low as the pre-experimental level. That the blood sugar curve should follow such a course might be due partly to the hyperglycaemic effect of the operative procedure.

Effect of stimulation with choline chloride

Choline chloride, which was injected in massive doses of from 110 mg. to 240 mg. over a period of 5 to 6 hours in eight cats, produced striking effects on the pancreatic gland. The acinous cells were almost entirely depleted of zymogen granules. About 80% of all the islands were surrounded by "halos." α -cell "precursors" were very numerous, being situated in groups near the islands. The continuous simple tubular structure of the gland was evident. At a certain point of its extent the dual nature of the acinous tubes is revealed, the apical part of the cells showing an exocrine character and the basal part having all the characters of endocrine cells. The anatomical continuity of the tubes is uninterrupted but their function changes. The picture is very like that presented in figure 2.

During choline injection the blood sugar level was moderately increased, in one case from 88 mg. to 113 mg. per cent (the first blood sample being taken before the operation), and in another case from 159 to 224 mg. per cent (the first blood sample being taken after the operation).

Section of the vagus nerves (with both suprarenals intact)

This operation was performed on three cats. In one case both vagi were cut in the chest below the heart and the animal was sacrificed and the histological material taken 40 days later. In the other two cats the vagi were cut below the diaphragm and the animals were sacrificed 21 days after the

operation. The first case presented the following microscopical picture (with which the other two were in agreement). The acinous tissue appeared as a monotonous background, the cells containing few zymogen granules. The islands were numerous and rounded in shape and contained a striking preponderance of α -cells; they were surrounded by "halos." No

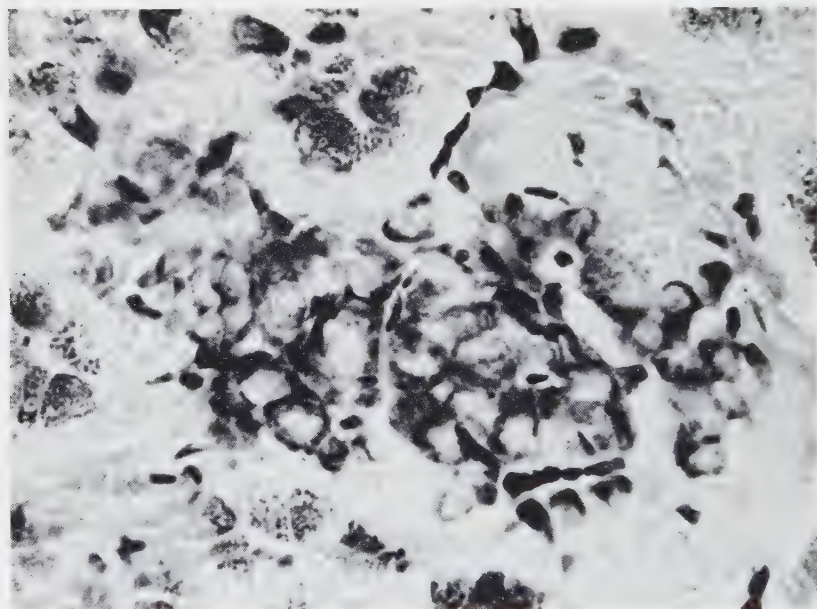


Fig. 4 An island from a case where the vagus nerves had been cut 40 days previously. The island is rounded in shape. There are numerous dark α -cells in the centre, while β -cells are at the periphery and not very prominent. The island cells are in close contact with the acinous cells. The "halo" is noticeable. The zymogen content of the acinous cells is diminished. Fixation: Bailey's solution. Staining: Bowie's Biebrich-scarlet, basic ethyl-violet. $\times 600$.

sharp demarcation between the island cell cords and the acinous tissue was noticeable. In some places the α -cells gradually merged with the acinous cells. β -cells were not very numerous in the islands and did not stand out prominently (fig. 4).

The blood sugar concentration was determined several times during the 40 days following the operation and showed no

deviation from the normal level. Thus the operation was performed on 3rd November. The blood sugar concentration on 17th November was 84 mg. per cent, on 21st November 82 mg. per cent, on 24th November 90 mg. per cent, and on 5th December 92 mg. per cent.

Effect of stimulation of the splanchnic nerves after removal of the suprarenals

In a series of five experiments the suprarenals were removed and the splanchnic nerves were stimulated rhythmically. In some of the experiments 6 to 7.5 mg. of ergotamine methan-sulphonate (Sandoz) were injected intravenously. The microscopical picture was very much the same in all cases. There was a definite exocrine secretion notwithstanding the short duration of the experiments, which sometimes did not exceed 1 hour. After stimulation of the splanchnic nerves, the islands tended to disappear. In the middle of the lobule, where the islands are usually situated, the capillaries were dilated, and the short, colorless cell cords were in intimate contact with the surrounding acinous tissue (compare fig. 10—Sergeyeva, '38). Under high power magnification the large accumulations of island cells were seen to have diminished—both α and β elements equally—but throughout the acinous tissue small groups of cells had appeared which closely resembled β -cells. Though the islands had decreased in size, they had increased in number and dispersion throughout the acinous tissue (fig. 5).

Soon after the completion of the operative procedure, which included removal of the suprarenals, splanchnic stimulation produced a marked rise of the blood sugar concentration, but after 2 or 3 hours of stimulation it began to fall rapidly.

Effect of removal of the abdominal sympathetic chains and ganglia and section of the splanchnic nerves

In three cats, in which this operation was performed first on the right side and then on the left side (the latter operation 1 month previous to the sacrifice of the animal), the pancreas

presented the following microscopic picture. The exocrine tissue contained a fair amount of zymogen granules. The islands had increased in size and become irregular in contour, branching out in various directions into the acinous tissue

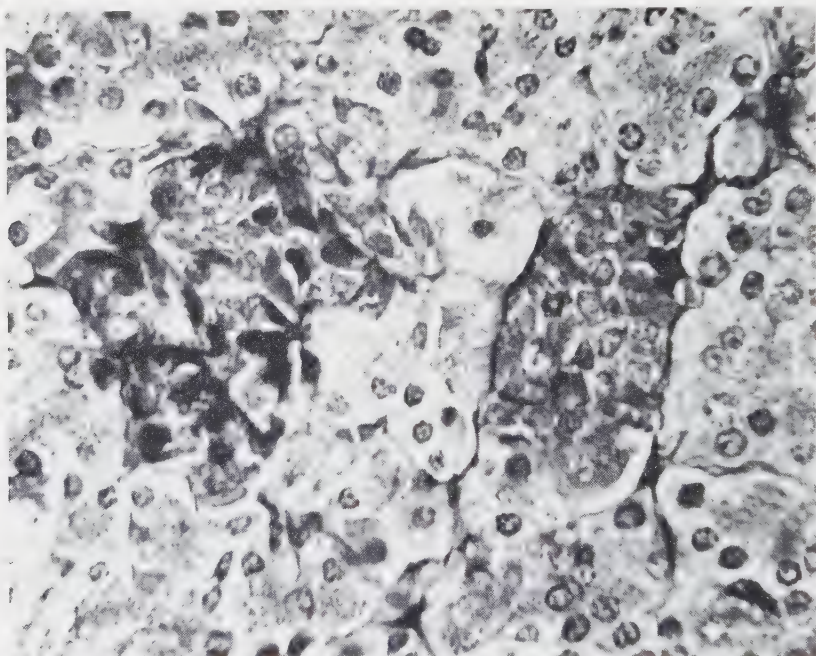


Fig. 5 Pancreatic gland of a cat when the splanchnic nerves have been stimulated electrically after the suprarenals had been removed. The small groups of dark cells consist exclusively of β -cells. Fixation: Bouin's fluid. Staining: Gomori's method. $\times 600$.

(fig. 6). Most of the islands contained a few α -cells but some of them none at all. At the periphery the island cell cords had acquired the same shape as the "rosettes" of surrounding acinous tissue. β -cells predominated in the islands and small groups of β -cells were scattered throughout the acinous tissue.

A systematic determination of the blood-sugar concentration was made in one animal, blood being taken from the ear vein. Before removal of the right sympathetic chain the blood sugar concentration was 81%. This operation was performed

on 20th December; the blood sugar concentration on 10th January was 84 mg. per cent, on 17th January 88 mg. per cent, on 24th January 106 mg. per cent, on 31st January 97 mg. per cent, and on 7th February 95 mg. per cent. The left sympathetic chain was removed on 9th February; the blood sugar concentration on 21st February was 99 mg. per cent and on 9th March 95 mg. per cent.

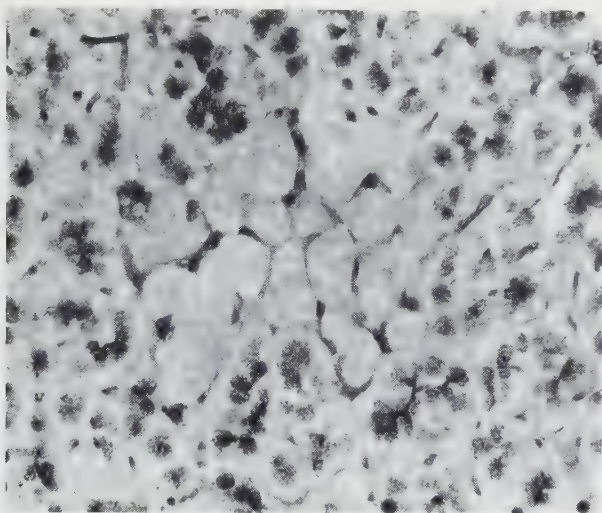


Fig. 6 Island from the pancreatic gland of a cat after removal of both abdominal sympathetic chains, etc. Note the irregular contour of the island. Almost no α -cells can be seen in the island, only β elements. There is very close continuity between the island cells and the acinous cells. A fair amount of zymogen is present in the acinous cells. Fixation: Bailey's solution. Staining: Bowie's Biebrich-scarlet, basic ethyl-violet. $\times 300$.

Effect of stimulation with adrenalin

In ten cats after adrenalin was administered over a period of 5 hours (altogether 24 cc. of 1:10,000 adrenalin being introduced), there was no microscopical evidence of exocrine secretion in the pancreas. The acinous cells were filled with granules. The islands, however, had increased in number and in size. The α -cells were very conspicuous and were situated

mostly in the middle of the islands, the β -cells as usual at the periphery.

Effect of stimulation of the splanchnic nerves with the suprarenals intact

After 4 to 5 hours of rhythmic stimulation of the splanchnic nerves (coil 12 to 8 cm.) the microscopical picture showed a definite exocrine secretion in the pancreas. "Halos" could be seen around 40% of all the islands (under low power). The size of the islands was increased, mostly owing to the increased numbers of α elements. "Precursors" of α -cells were very numerous in some cases. Under this kind of stimulation the islands were observed to have acquired a peculiar shape, appearing as "coiling, snake-like" columns of elongated cells indistinguishably merging in many places with the surrounding acinous tissue (see fig. 9—Sergeyeva, '38). During this type of experiment the blood sugar concentration was greatly increased, i.e. to more than three times its previous value.

Effect of stimulation of the vagus nerves after removal of the abdominal sympathetic nerves

In one animal in which first the right, and later the left abdominal sympathetic nerve had been removed, the vagi were stimulated with an induction current over a period of 2 hours 1 month after the second operation. A sample of the pancreas after stimulation showed the following microscopical picture. The zymogen granules in the acinous cells were decreased in number; the island cell cords were coiling and disfigured. At the periphery of the islands, groups of endocrine cells could be seen, shaped like the "rosettes" of the acinous cells. There was very close continuity between the exocrine and the endocrine parts of the gland.

In the control sample of pancreas no α -cells were visible in the islands. In the sample taken after 2 hours of vagal stimulation α -cells could be seen in the islands. However, they stained pale and were not conspicuous. The number of β -cells was greatly increased. The blood sugar concentration rose

moderately during the experiment, this being attributable chiefly to the operative procedure.

*Effect of stimulation of the splanchnic nerves after
section of the vagus nerves*

In one cat, in which the vagus nerves had been cut 40 days previously, the splanchnic nerves were stimulated during a

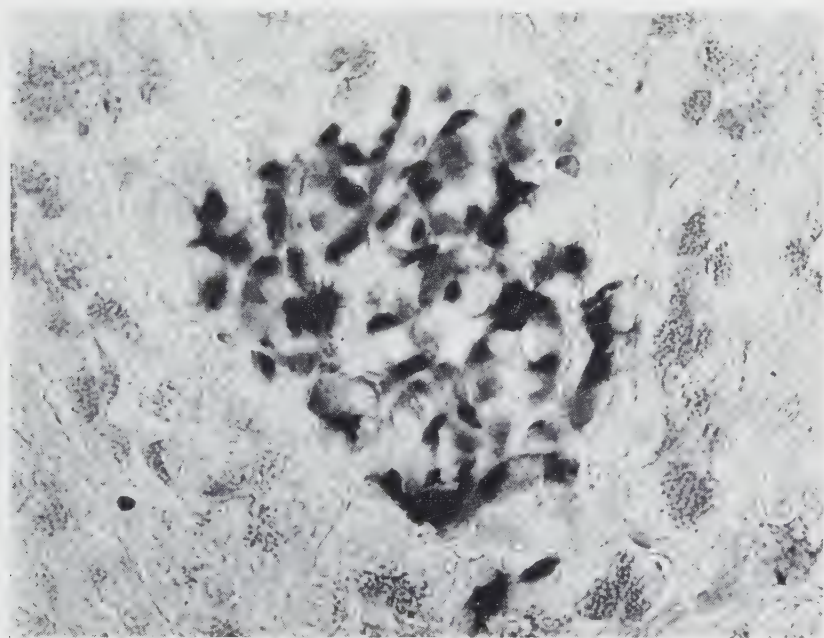


Fig. 7 From the same animal as in figure 4. The sample was obtained from the pancreatic gland after 3 hours of splanchnic nerve stimulation. The island is rounded in shape. The α -cells are greatly increased in number as compared with figure 4. There is very close continuity between the endocrine and exocrine tissues. The zymogen content of the acinous cells is diminished. The "halo" is clearly visible. Fixation: Bailey's solution. Staining: Bowie's Biebrich-scarlet, basic ethyl-violet. $\times 600$.

period of 3 hours. After the stimulation, the α -cells were greatly increased in number in the islands; β -cells were present but were not very numerous. The acinous cells were almost entirely depleted of zymogen granules, but "halos" could be seen around the islands (fig. 8). The blood sugar level gradu-

ally fell during this experiment. Before the experiment it was 124 mg. per cent, 2 hours later 103 mg. per cent, and 3 hours later 80 mg. per cent.

Feeding with cream

In two cats which were sacrificed 3 hours after being fed with cream, the microscopic picture of the pancreatic gland (fig. 2) closely resembled that obtained after stimulation of the splanchnic nerves with the adrenals intact. The acinous cells were depleted of zymogen granules to the utmost limit. The blood vessels were dilated. The islands were tremendously increased in number and size and were composed chiefly of α -cells. The island cell groups resembled the rosettes of acinous cells in contour. It seems that transformation of the acini into island tissue was proceeding so quickly that the newly formed island cell groups still retained their alveolar shape. No compression of either type of tissue was noticeable.

Ligation of the main pancreatic duct

In two cats in which the pancreatic duct had been ligated for 2 weeks before the animals were sacrificed, the pancreas presented the following microscopic picture. Clumps of atrophied acinous tubules were surrounded by strands of connective tissue. Remnants of zymogen granules could be seen in the cells. In the centre of these clumps were "cords" of island cells, containing mostly α -cells; the number of β -cells in them was greatly diminished. Scattered α elements persisted throughout the atrophied acinous tubules.

In one case (a dog), from which samples of the gland were obtained after 2½ months' ligation of the main pancreatic duct, β -cells were almost entirely absent, only α -cells being present. In a case where ligation of the duct was performed in conjunction with cutting of the vagus nerves below the diaphragm, the histological material was taken 1 month after the operation. Microscopic examination showed that the acinous tubules were not completely atrophied and contained a fair

amount of zymogen granules. This might be attributed to the existence of accessory ducts which were not ligated or to re-establishment of the connection between the duct system and the intestine. The islands were large and coiling, and they contained numerous α -cells. Many of the islands were situated near the margin of the lobules. The picture presented by the coiling shape of the islands and their greatly increased content of α -cells closely resembles that obtained when the splanchnic nerves were stimulated with the suprarenals intact. The blood sugar level was normal during the whole time of observation. Before the operation (performed 17th January) it was 92 mg. per cent; on 31st January it was 93 mg. per cent, on 7th February 95 mg. per cent, on 14th February 93 mg. per cent, on 21st February 110 mg. per cent, and on 23rd February 102 mg. per cent.

DISCUSSION

The effects observed in the α - and β -cells after stimulation or destruction of the sympathetic or parasympathetic nerves to the pancreatic gland are classified in table 1. The α -cells were increased in number when the sympathetic nervous system exerted a predominant effect on the pancreas (point 1), i.e., when the vagal influence had been eliminated for several weeks through section of the vagi (1 a), after injection of adrenalin (1 b), or after stimulation of the splanchnic nerves with the suprarenals intact (1 c and d). An exception to this rule is presented by experiment 3 d, where the α -cells, which were not visible in a control piece of pancreas taken from a sympathectomized cat, appeared again after vagal stimulation.

There was a diminution in the number of α -cells after removal of the abdominal sympathetic nervous supply (point 2).

An increase in the number of β -cells took place after splanchnic nerve stimulation under special conditions (point 3 a and b). In these cases we actually were dealing with the effect on the pancreas of stimulating the cholinergic and not the adrenergic fibres of the splanchnic nerves (Babkin, Hebb and Sergeyeva, '39). Similarly, when the sympathetic influence on the pancreas was withdrawn for a long time (point 3 c), the effect of the

parasympathetic nervous system on the gland was predominant and resulted in an increase of the β -cells.

In contrast with these data, prolonged stimulation of the vagus nerves (point 5 a) or administration of choline in massive doses (5 b) increased the numbers of both α - and β -cells. Moreover under the influence of these stimulations marked changes took place in the islands: vacuoles appeared in the endocrine cells, the outlines of the cell cords became indistinct and some of the islands appeared to be greatly distorted.

TABLE 1

Changes in the numbers of α - and β -cells in the islands of Langerhans as a result of different procedures

1. α -cells increased in number by:
 - (a) Section of vagus nerves (survival experiment).
 - (b) Injection of adrenalin.
 - (c) Stimulation of splanchnic nerves with suprarenals intact.
 - (d) Stimulation of splanchnic nerves several weeks after section of vagus nerves.
 - (e) Feeding with cream.
2. α -cells decreased in number by:

Extirpation of abdominal sympathetic chains, including the ganglia, and section of splanchnic nerves (survival experiment).
3. β -cells increased in number by:
 - (a) Stimulation of splanchnic nerves with suprarenals removed.
 - (b) Stimulation of splanchnic nerves after ergotamine administration.
 - (c) Extirpation of abdominal sympathetic chains and ganglia and section of splanchnic nerves (survival experiment).
 - (d) Stimulation of vagus nerves several weeks after removal of abdominal sympathetic chains, etc. (α -cells not visible in the islands in a control piece of pancreas but appeared after stimulation of the vagi.)
4. β -cells decreased in number by:

Ligation of main pancreatic duct.
5. Both α - and β -cells increased in number by:
 - (a) Stimulation of vagus nerves.
 - (b) Injection of choline.

It is clear therefore that stimulation or ablation of the autonomic nerves has a marked effect on the endocrine tissue of the pancreatic gland. The interpretation of the data here described presents great difficulties. How, one may ask, do the

autonomic nerves act on the endocrine cells? There are several possibilities to be considered:

(a) The nerves may be true secretory nerves acting directly on the α - and β -cells.

(b) The nerves may act indirectly on the endocrine cells by affecting the vascularization of the pancreas.

(c) The nerves may act indirectly on the endocrine tissue of the pancreas by stimulating the exocrine secretion of the organ and thus diminishing the pressure which the acinous cells loaded with granules exert on the islands, or perhaps in acute experiments by causing diffusion of the acinous secretion with its enzymes over the endocrine cells.

(d) The nerves may have a trophic (nutritional) function in relation to the endocrine cells of the pancreas.

Let us discuss each of these possibilities.

(a) By a true secretory nerve is meant a nerve which is able to cause secretion (i.e., passage into the cells of water and crystalloids from the blood and discharge of the specific organic material elaborated in the cells) independently of all other factors, such as increased blood flow through the gland or contraction of the muscular sheaths which may surround a gland. Can we apply this criterion to the autonomic nerves of the pancreas and ascribe to one of them the role of a true secretory nerve for the elements of its endocrine tissue? Although the secretory nerves for the endocrine glands may act somewhat differently than those for the exocrine glands, nevertheless they must have some traits in common. The results obtained in the present histo-physiological study neither prove nor disprove the supposition that one of the pancreatic autonomic nerves is a secretory nerve. The diminution, or still more the increase, of α - or β -cells following the various experimental procedures described above cannot be regarded as proof of the secretory nature of the autonomic nerves, nor indeed can any of the other changes observed by us in the endocrine tissue, such as the tendency of the islands to disappear, the tendency of the β -cells to disappear from the islands and appear dispersed throughout the gland, distortion

of the islands, and so on. Again, even during very prolonged stimulation of the vagus or splanchnic nerves (with the suprarenals removed) the blood sugar was never observed to fall below the normal level. Similar observations were made on animals which lived for weeks after extirpation of the abdominal sympathetic or the vagus nerves. The problem of the secretory nerves for the endocrine tissue of the pancreas thus requires further investigation.

(b) Very great attention should be paid to vascular changes in the gland. From table 1 it may be seen that in almost all cases where the influence of the sympathetic nervous system predominated (point 1) there was an increase in the number of α elements and a diminution in the number of β elements. A diminution of α -cells, on the contrary, was observed when an animal was deprived of its sympathetic abdominal innervation for a long period of time (point 2).

(c) The enlargement of the islands and the appearance of scattered α - and β -cells throughout the acinous tissue under certain conditions of stimulation could hardly be attributed to decrease of the acinous cells and elimination of compression of the endocrine tissue by acinous cells loaded with zymogen granules, because the endocrine cells in the control pieces of pancreas, i.e. before stimulation, did not appear to be compressed. Another important question is whether there is diffusion of the exocrine secretion with its various enzymes into the surrounding tissue. It is particularly noticeable in the cat that stimulation of the vagus or the splanchnic nerves produces practically no visible secretion which can be collected from the duct. However, as was demonstrated previously (Sergeyeva, '38), the acinous cells under these circumstances do not contain granules, but the secretory material is seen to be deposited in the ducts and ductules. A decrease in the number of β -cells was also observed after ligation of the pancreatic ducts.

(d) The problem of trophic-nutritional nerves is far from being solved. If such nerves exist, then it is to be expected that abolition of their effect—as, for example, when the abdominal sympathetic nervous supply is removed, with conse-

quent decrease in the number of α elements—would influence the normal metabolic processes in certain groups of cells. Unfortunately we know too little about trophic nerves to draw any conclusions as to the role they might have played in our experiments.

Therefore at the present stage of this research no definite conclusion can be drawn regarding the intimate relationship between the endocrine tissue of the pancreas and the autonomic nerves, although the results of stimulation of these nerves or abolition of their effect were clearly demonstrated in the present work.

It is interesting to note that many previous investigators and recently Woerner ('38, '39) and Gomori, Friedman and Caldwell ('39) (for literature, see these papers) have observed changes in the β -cells and also in the α -cells of the islands of Langerhans in guinea pigs as a result of continuous administration of glucose.

There is another aspect of this problem which should be mentioned. That the pancreatic tissue is of two types—exocrine and endocrine—is admitted by most investigators; however, opinions differ widely concerning the relation between these tissues. Many investigators are inclining to the view that these two types of tissue are not entirely dissimilar and that under certain circumstances they might be interchangeable (for literature, see for example Kohn, '30). The following facts observed in the course of the present investigation seem to favor the theory that exocrine tissue may be transformed into endocrine tissue, and vice versa: the appearance of transitional cell forms under various experimental conditions; the appearance of α -cell "precursors" in many cases; distortion of the islands, in which state the continuity of the tubular structure of the acinous and endocrine tissues is revealed (such a condition of the islands was especially noticeable when choline was administered in massive doses); increase or decrease in the numbers of α - and β -cells in the islands and appearance of scattered α and β elements among the acinous cells under various circumstances. There is no doubt, however, that much

more work must be done before definite conclusions can be reached concerning the actual relations between the exocrine and the endocrine tissues of the pancreas.

SUMMARY

1. Stimulation of the vagus or the splanchnic nerves with an induction current in acute experiments, or aseptic removal of these nerves in surviving animals and elimination of their influence on the abdominal viscera for a considerable time, produced changes of various kinds in the endocrine tissue of the pancreatic gland of the cat.

2. Microscopic examination of samples of the pancreatic gland revealed an increase in the number of α -cells in most cases where the influence of the sympatho-adrenal system predominated, for example, under stimulation of the splanchnic nerves with the suprarenals intact or administration of adrenalin, or in survival experiments following section of the vagi. On the other hand, the number of α -cells was found to be diminished when the pancreas had been deprived for some time of the influence of the sympatho-adrenal system.

3. An increase in the number of β -cells was noted when the cholinergic fibres of the splanchnic nerves had been set into activity, or when the parasympathetic nervous system had exerted a predominant influence on the pancreatic gland as a result of previous removal of the abdominal sympathetic nervous supply.

4. Under some more complicated experimental conditions, changes also took place in the number of endocrine elements. Thus a great increase in the number of α -cells was observed in the pancreas of a cat several hours after feeding with cream.

5. α - and β -cells were found not only accumulated in the islands of Langerhans but also scattered throughout the acinous tissue in small groups or even singly. Under certain conditions these scattered endocrine elements undergo the same changes as the cells in the islands of Langerhans.

6. Some morphological proofs are presented of the possible transformation of acinous tissue into endocrine tissue and vice versa.

In carrying out this work I have received much helpful advice from Professor P. Masson, Professor B. P. Babkin and Dr. L. C. Simard, which I gratefully acknowledge. I wish to express my thanks to the Banting Research Foundation for the award of a grant which made this research possible.

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AN ABERRANT PANCREATIC NODULE ARISING ON THE NECK OF A HUMAN GALL BLADDER FROM MULTIPLE OUTGROWTHS OF THE MUCOSA

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ONE TEXT FIGURE AND TWO PLATES (SEVEN FIGURES)

The term, "aberrant pancreatic tissue", might well be defined as pancreatic tissue outside of the usual location, and without the relation of continuity or of vascularization with the major pancreas. This definition excludes annular pancreases and pancreatic bladders not originating from an accessory pancreas.

REVIEW OF LITERATURE

Aberrant tissue usually appears as a small, firm, nodular formation of whitish opaque color, and is not infrequently associated with other anomalies. In over 90% of the cases this anomaly is found in the foregut, as in the wall of the stomach or in the small intestine, and in greater frequency with closer proximity to the normal pancreas. The location may be submucous, intramural, subserous or invading all three layers, and may even extend to the liver, as reviewed by Boyden ('22), but it is most often submucous. Aberrant pancreatic tissue may also be found in the adjacent peritoneal formations (nine cases), in the wall of the gall bladder (three cases, as reviewed by Poppi, '35), and in the capsule of the spleen (three cases, as reviewed by Branch and Gross, '35), but has never been found in the wall of the colon or the rectum.

From an analysis of the world's literature on human aberrant pancreases, the author was able to record reviews and

individual reports of 347 cases from authors as follows: Az-zolini ('35), Best ('35), Best and Bowers ('34), Black and Packard ('38), Branch and Gross ('35), Branscheid ('38), Ciceri ('35), Cruickshank ('39), Danzis ('38), Donovan and Aquirre ('36), Gilbert and Bordet ('35), Heilig ('37), Hill and Cohn ('37), May ('37), Mazzini ('38), Nakamura ('36), Picco ('38), Poppi ('35), Stohr and Kögler ('36), Udaondo and Jaca ('38), Ugelli ('35), Whiteside ('37) and Zaslavskiy ('38).

Poppi ('35), reviewed the reports of six different pathologists from 4,076 autopsies and found the frequency of aberrant pancreatic tissue to be 1.89%. In some cases such tissue occurred in more than one place in a given individual.

From the above three cases of aberrant pancreatic tissue found in relation to the human gall bladder, Otschkin ('16) as quoted by Poppi ('35), was the first to have reported a case of aberrant pancreatic tissue in the wall of the cystic duct of the gall bladder. The second case was removed at operation from a 40 year old woman, by von Hedry ('24), who described a small and contracted gall bladder with marked thickening by inflammation and fibrosis, with most of the mucosa destroyed and containing purulent contents with several faceted cholesterol stones. At the border of the middle and proximal thirds of the gall bladder, about 2 cm. from the beginning of the cystic duct, a resistant formation was palpable in the outer fibrous layer of wall and was yellowish on section with a transverse diameter of 10 mm., in thickness 7 mm., and in length 8 mm. The microscopic structure revealed typical pancreatic tissue with acini, large and small ducts in interstitial tissue, also two typical islands of Langerhans and richly vascularized. The third case was reported by Cogniaux ('28), as case 4, of his series of aberrant pancreases, for which cholecystectomy was performed in a 39 year old woman. In this specimen there was chronic inflammation of the gall bladder and hypertrophy of the glands and discrete infiltration of the mucosa, with a small aberrant pancreas beneath the mucosa of the gall bladder. It formed three or four lobules with nor-

mal appearing acini but without excretory ducts or visible islands of Langerhans.

Aberrant pancreatic tissue has also been found in the wall of the gall bladder in several of the lower animals. Mann ('22), Higgins ('26, '30), have reported three such cases in the dog. It is interesting to note in this connection the incidence of portal lobes of the ventral pancreas, either alone or associated with pancreatic bladders. Boyden ('25) made a survey of 2600 cats and examined an equal number of adult pigs, sheep and cattle. In the cattle no such anomalies were found; in the pigs there were fifteen portal lobes, none of which, however, extended beyond the porta hepatis; in the sheep three cases were found paralleling the course of the bile duct from duodenum to gall bladder; and in cats there were forty portal lobes, fourteen of which extended to the neck of the gall bladder.

In these 2600 cats there were also five specimens with a pancreatic bladder, one of which (case 4, fig. 12, loc. cit.) opened distally into the neck of the gall bladder. This fistulous connection undoubtedly arose early in embryonic life, and the specimen proves that in mammals portal lobes may acquire a secondary connection with the biliary tract. But since no accessory pancreas belonging solely to the gall bladder was found in these 2600 cats—the species in which the portal lobes of pancreas are most numerous—it would seem that in mammals, nodules on the neck of the gall bladder must arise *in situ* rather than by migration of pancreatic tissue through the duodeno-hepatic ligament.

OBSERVATIONS

The specimen to be described was obtained from a gall bladder removed at operation¹. The long narrow vesicle

¹ The patient, Mrs. E. C. A., age 34 years, entered Denver General Hospital, April 6, 1937. She was well until the previous October, when, following menstrual periods, she began having attacks of upper abdominal pain which radiated to her back and which had no relation to the taking of food. Since her illness she had lost 24 pounds. There was slight upper abdominal tenderness present to palpation. She had a cholecystectomy on April 16, 1937, with an uneventful convalescence and was discharged from the hospital May 1, 1937.

measured 8×2 cm., unopened, and contained greenish bile with twelve small, irregular, cholesterol pigment stones partly impacted in the ampulla (the folded and dilated neck of the gall bladder). On the anterior side of the neck, apparently filling the space between the mucosa and the serosa of the organ, was a relatively large nodule 5×10 mm. in size. Upon microscopic section this mass was found to consist primarily

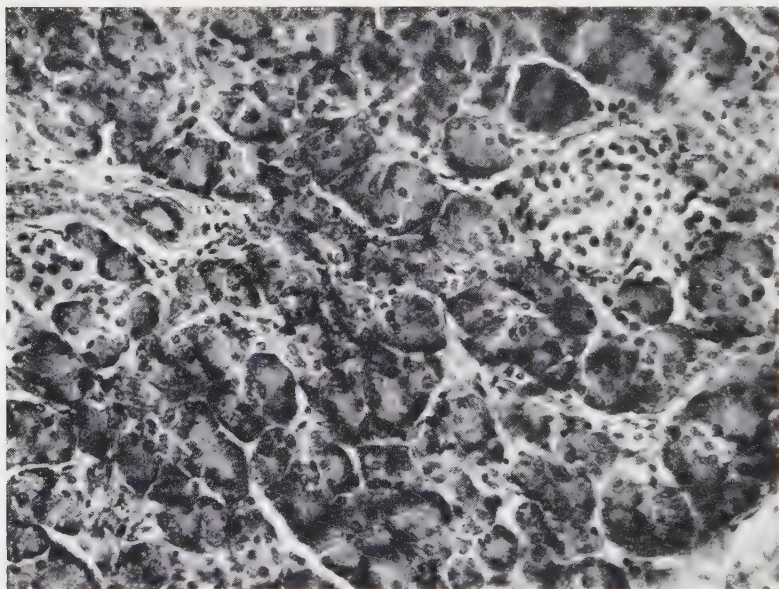


Fig. 1 High power photomicrograph of that lobule of aberrant pancreatic tissue which lies half way between C and Ser. in figure 2. ($\times 227$). Intralobular duct to left, island of Langerhans to right.

of pancreatic acini, a few islands of Langerhans (fig. 1), dilated ducts, and cervical glands. In addition, the wall of the gall bladder exhibited slight superficial ulceration of the mucosa and infiltration of lymphocytes.

As viewed in section (fig. 2) the arrangement of tissues is as follows: Lining the cavity of the neck is a distended mucosa somewhat fragmented by postoperative changes. Its basal

crypts, here and there, give rise to mucous glands. In some places these merely line up as a single row of end pieces in the dense subjacent tunica propria. In other stretches they expand into voluminous masses of tubulo-alveolar glands, five or six rows thick, lying in an areolar tunica propria (fig. 4). These are the mucous glands which are peculiar to the neck of the human gall bladder (cf. Halpert, '27, fig. 6). Some of these masses are markedly distended (fig. 2), suggesting the condition reported by Cogniaux ('28, case 5) in which aberrant pancreatic tissue in the wall of the pylorus of the stomach was sufficiently dilated to obstruct the lumen of the pylorus. Associated with these glands of the tunica propria are subepithelial patches of aberrant pancreatic tissue to be described later. Peripheral to these glands and subepithelial pancreatic lobules lies the greater mass of pancreatic tissue, interspersed with bands of smooth muscle, although the bulk of the pancreatic tissue lies in the peri-muscular tissue. It is possible that this main mass empties by the large cluster of ducts shown in figure 3, but owing to the fact that serial sections were not made until after part of the blocks had been discarded, it was not possible to trace these ducts.

Of special interest are the smaller patches of accessory pancreatic tissue located next to the lumen of the gall bladder. Two such masses are shown in figure 2, the upper of which appears also in figure 4. The ducts which drain these smaller patches may be traced to the cervical glands of the gall bladder. Figures 5-8 show two such places: D¹ (fig. 6) is a small duct buried in pancreatic acini. D¹ (fig. 5) shows the same duct about to empty into a cervical gland of the gall bladder. A second site of origin, 75 μ below, is shown in figures 7 and 8. Therefore, whatever the drainage of the peripheral mass of the nodule may have been, figures 5-8 demonstrate that the pancreatic tissue of the tunica propria arises by multiple origin from the glands of the gall bladder. So far as is known, this is the first instance of its kind.

DISCUSSION

The theories of origin of aberrant pancreas are numerous and diverse, and they overlap to such an extent that it is difficult to group them into categories. All of them necessarily attempt to meet the fact that the accessory pancreas arises within the embryonic territory lying between the cardiac antrum and the vitelline duct, i.e., the abdominal portion of the foregut, including its derivatives, the spleen and biliary duct system. In a human embryo of $3\frac{1}{2}$ weeks this territory is compressed into a short, somewhat flattened, trumpet-shaped segment surrounding the anterior intestinal portal. This might also be defined as the segment which contains the ring of Weber—a narrow zone destined shortly, to give rise to a dorsal and two ventral pancreatic outgrowths. In other words, the presumptive area for the development of pancreatic tissue may be coextensive with the larger segment, although ordinarily this potency is suppressed in the zones immediately above and below the ring of Weber. In the course of the next few weeks, as the parts of the gut tract are differentiating, this segment will expand enormously in length, thus giving wide distribution to cells originally adjacent to the ring of Weber.

A modification of this theory would assume that the normal anlagen are carried away from the original site because they are slightly out of position. Thus Warthin ('04), states, "It is probable that accessory pancreatic tissue is formed from lateral budding of rudimentary pancreatic ducts as they penetrate the intestinal wall, the mass of pancreatic tissue thus formed being snared off and carried by longitudinal growth of the intestine, either upwards or downward."

The theory advanced by Beutler ('21) and contemporary German pathologists, that the accessory pancreas is an atavistic phenomenon—based on the occurrence in fishes of widely scattered primordia—may also be considered as merely another way of stating the facts noted above, since it is yet to be

proved that the pancreatic elements in fishes do not arise from a common zone in the embryo².

Another hypothesis considers that such aberrant glands represent congenital heteropias or metaplasias due to environmental modification of cells not destined to give rise to pancreas (Taylor, '27, et al.). But, since these occur only in the territory of the foregut, one is obliged to assume some embryonic predetermination.

A second line of attack is represented by the work of Horgan ('21), who postulates non-inflammatory adhesions in fetal life between the definitive pancreas and contiguous viscera. (Lubarsch, '24, would assume that the adhesions resulted from inflammation.) Certain experimental support for this theory has recently been furnished by Schofield ('38), who transplanted the pancreas of salamander embryos to the body cavity of the same species of embryo. Such grafts grew through the walls of adjacent portions of the small intestine, there establishing connection with the lumen of the gut, but never entered the colon, even when planted along side it. But it should be pointed out that amphibian tissues have capacities for growth and regeneration not possessed by warm-blooded vertebrates and that if such processes occurred in mammalian embryos someone should have encountered one or more stages in the development of such an adhesion. However, the fact remains that examination of human embryonic material has provided only one direct observation, and that was not corroboratory of this view. F. T. Lewis ('12b) found an accessory pancreas developing from an elongated epithelial bud in the wall of the stomach of a 19 mm. human embryo (see figs. 7 and 12 of that article; also the account of 1912 a). Similar buds had previously been found by Lewis and Thyng ('08) along the small intestine of pig embryos 10 to 20 mm. long;

²Of special interest, in connection with the present article, is the occurrence in myxinoid fishes of pancreatic lobules that are embedded in the serosa of the gut, and that empty separately into the cystic duct (Wiedersheim, '09, p. 511).

and from a comparable site, in the adult cat, Boyden ('22) found an accessory pancreas extending from the duodenum into the porta hepatis, the distal end of which formed a pancreatic bladder. This undoubtedly arose from such a diverticulum as the one shown by Thyng ('08), in his figure 2. In this connection it is of interest that Poppi ('35) describes twenty-five cases (9.36%) in which an accessory pancreas in man was found at the tip of some diverticular formation of the gastroenteric tract. It would thus appear that one must return to the theory of Zenker (1861), which assumed the origin of accessory pancreases to be from accessory pancreatic anlagen, and to conclude with Lewis ('12 a) that such primordia arise from elongated epithelial buds in the wall of the fore-gut.

Finally, this case illustrates the importance of routine microscopic sections of nodules from the neck of the gall bladder, which might easily be mistaken for a lymph node.

It is believed by the author that the accessory pancreatic tissue might well be the cause of the pathologic changes in the gall bladder in this case as well as cases of von Hedry ('24) and Cogniaux ('28), possibly also in the other cases where pancreatic tissue has been overlooked. Evidence for this is supplied by the work of Colp and Doubilet ('38), who have found that reflux of pancreatic ferments into the biliary tract, following spasm of the sphincter of Oddi, may produce either a nonperforative bile peritonitis, or an acute cholecystitis.

The author wishes to express his gratitude to Doctor Edward A. Boyden for valuable assistance in the preparation of this article.

SUMMARY

A pancreatic nodule on the neck of a human gall bladder is described.

Investigation of the literature reveals no previous case in this country, of aberrant pancreatic tissue in the wall of the human gall bladder, and only three cases in the foreign literature.

This case is of special interest in that the nodule appears to have arisen from multiple outgrowths of the cervical glands.

It is suggested that such aberrant pancreatic tissue in the neck of the gall bladder may be a cause of pathologic changes in this organ.

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PLATE 1

EXPLANATION OF FIGURES

Low power photomicrographs illustrating character and arrangement of tissues in a specimen removed at cholecystectomy from a female patient, 34 years old.

2 Transverse section through neck of gall bladder and appended nodule of pancreas ($\times 6.5$). Ser., peritoneal surface of gall bladder; Lumen, cavity of gall bladder, mostly denuded of epithelium; C, area shown under higher magnification in figure 4, comprising typical cervical glands from which the subadjacent lobules of pancreas in the lamina propria are arising; B, area shown under higher magnification in figure 3, containing what is possibly the duct of the principal mass of the nodule, i.e., the part lying mainly in the perimuscular layer of the gall bladder wall.

3 Higher power of area shown at B in figure 2 ($\times 60$). This shows two clusters of ducts lying between the denuded tunica mucosa and the muscularis. The set nearest the lumen are ducts of cervical glands. A comparable cluster may be seen in figure 2, $\frac{1}{2}$ inch above area B, just under the denuded mucosa. The lower set probably represents the principal excretory duct of the perimuscular portion of the aberrant pancreas.

4 Higher power of area shown at C in figure 2 ($\times 60$). At the left is the mucosal surface, mostly denuded of epithelium. Ruga, denuded connective tissue core of epithelial fold; crypt, basal pocket of mucosa. Note transition (above) to tubulo-alveolar glands and (below) to lobule of pancreas lying in the subepithelial lamina propria. A comparable, subepithelial lobule may be seen $1\frac{1}{2}$ inches below C, in figure 2; Dist. duct, distended duct, the connections of which are not known. Note that it is surrounded by scattered muscles which, at a different level, may have caused its distention. Note a somewhat similar distended group of glands in figure 2, $\frac{1}{2}$ inch below C; Leuc., infiltrating lymphocytes, indicating mild degree of inflammation.

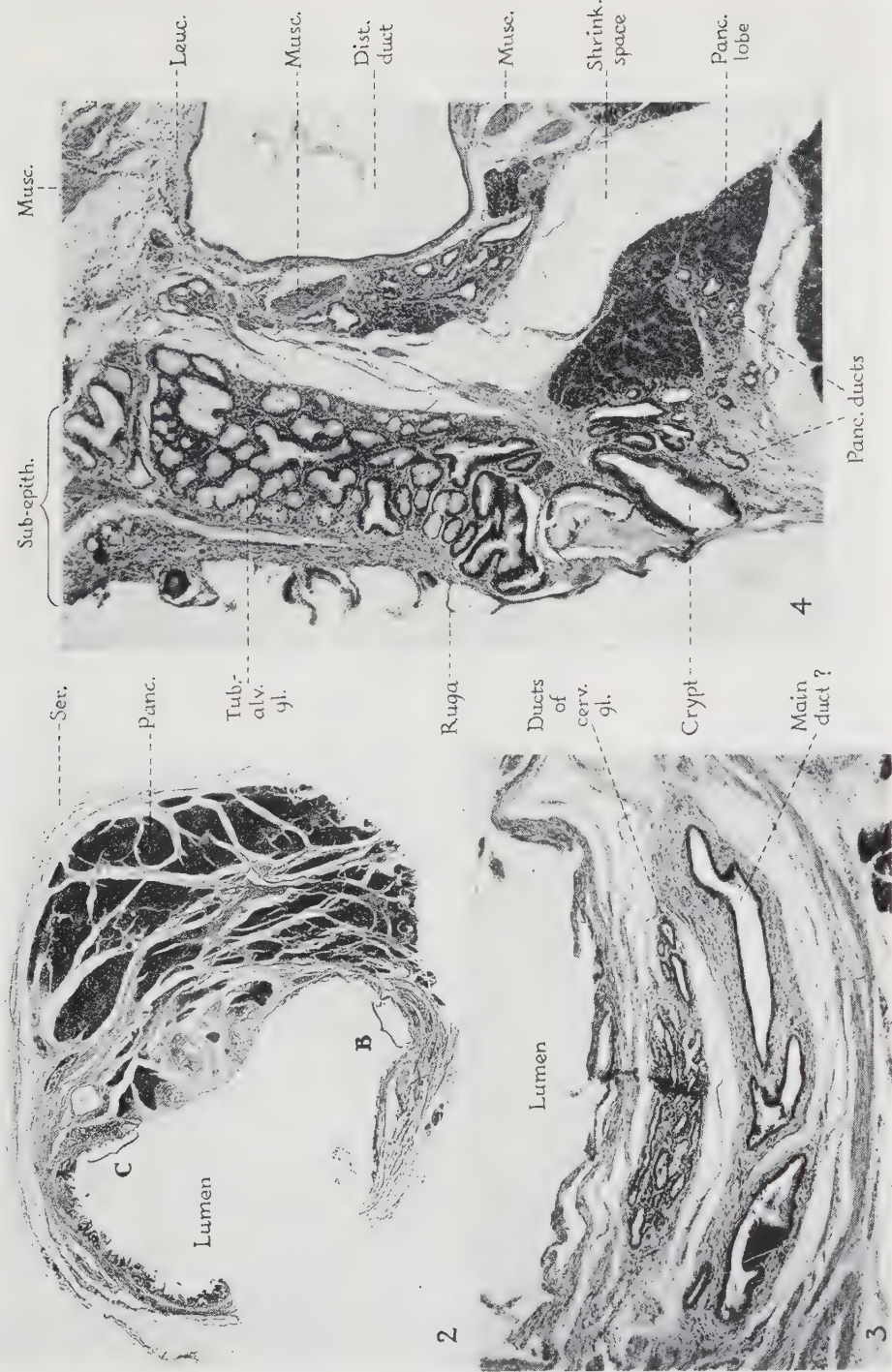


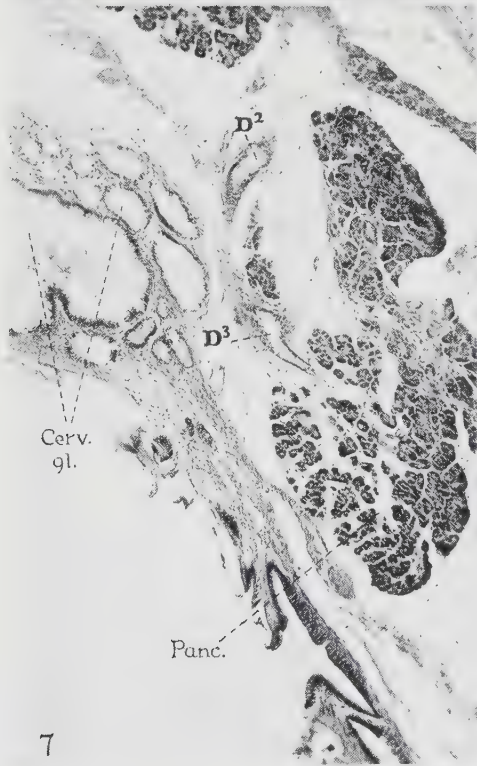
PLATE 2

EXPLANATION OF FIGURES

Two pairs of photomicrographs showing the origin of a sub-epithelial lobule of the aberrant pancreas from cervical glands of gall bladder ($\times 85$).

5 and 6 Two sections, 25μ apart, from a deeper level of the specimen than that shown in figure 2. The position on the circumference of the cavity corresponds to an area $\frac{1}{2}$ inch below the letter C in figure 2. Lumen, cavity of gall bladder mostly denuded of mucosal epithelium, except in crypts; D¹ (fig. 5), pancreatic duct arising from cervical gland (at left) and about to enter pancreatic lobe (at right); D¹ (fig. 6), same duct 25μ lower down, entering lobule.

7 and 8 Two sections, 25μ apart, the upper of which is 75μ below that of figure 6. D² and D² (fig. 7), pancreatic ducts about to enter lobule; D² and D³, (fig. 8) the same within the lobule.



VARIATIONS IN THE FORMATION OF THE SPLANCHNIC NERVES IN MAN

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Various standard text-books of human anatomy give essentially similar descriptions relative to the formation of the greater, lesser and least or lowest splanchnic nerves. The description given in Gray's Anatomy, twenty-third edition ('36), may be cited as typical: "The greater splanchnic nerve is formed by branches from the fifth to the ninth or tenth thoracic ganglia. . . . The lesser splanchnic nerve is formed by filaments from the ninth and tenth, and sometimes from the eleventh thoracic ganglia. . . . The lowest splanchnic nerve arises from the last thoracic ganglion."

From observations on dissections covering a period of years the authors have been repeatedly impressed with the apparent frequency in the variation of the gross origin of the splanchnic nerves as compared with the so-called "normal" mode of origin described in the standard text-books. So marked is this variation that it was considered sufficiently important, from a morphological as well as surgical point of view, to determine the average pattern of formation and the types of variation found in 100 cadavers. A review of the literature fails to reveal any published data bearing on this subject.

The nerves were dissected out and diagrammatic sketches of them made by students in the Human Anatomy Laboratory, all dissections and sketches being carefully checked and verified by the authors. Only those bodies were used in this study in which both sympathetic trunks and their splanchnic branches were intact and not damaged by faulty dissection or pleural adhesions. No records were kept of the color, sex or age of

the bodies, since the primary objective of this study was to determine the types of variation in gross origin of these nerves normally encountered in the dissection room. It may be added, however, that the majority of the cadavers in this laboratory are adult males, at least 65% of whom are colored.

ANALYSIS OF DATA ON SPLANCHNIC NERVES

For obvious reasons the mass of data obtained in this study are summarized in the following tables. Specific detailed information bearing upon the material collected will gladly be forwarded to any one interested upon request.

The first important consideration to be dealt with in this study concerns the total number of sympathetic ganglia which gave origin grossly to each type of splanchnic nerve. It was found that the greater splanchnic nerves in this series could be classified into seven groups according to the number of ganglia from which they arose; the lesser splanchnics into three groups and the least splanchnics into two groups. The number of nerves falling into each group, on both right and left sides of the body, is shown in table 1. It should be emphasized that the highest percent of greater splanchnic nerves arose from only three ganglia, as compared to five or six according to the typical text-book description; that the highest percent of the lesser splanchnics arose from only one ganglion rather than from two or three and, as should be expected, that the highest percent of least splanchnics arose from only one ganglion. Moreover, attention should be called to the marked lack of bilateral symmetry in origin of these nerves. Thus the greater splanchnic nerve arose bilaterally from the same number of ganglia in only 12% of the bodies; the lesser splanchnics in 35% and the least splanchnics in 79%. It is of interest to note that in 5% of the bodies the greater splanchnic nerve arose from only one ganglion, the frequency of occurrence being greater on the left side. For the most part, however, the distribution of these classes of nerves, as based on number of ganglia of origin, is of almost equal frequency on the right and left sides of the body.

The next important consideration is that of the frequency of gross origin of the splanchnic nerves from specific thoracic ganglia. Data bearing on this point are summarized in table 2. It can be observed from this table that the specific ganglia from which the greater splanchnic nerve may arise are four to twelve, inclusive; those from which the lesser splanchnic may arise are seven to twelve, inclusive and those from which the least splanchnic may arise are eleven and/or twelve. The apparent discrepancy between the results summarized in

TABLE 1

Classification of splanchnic nerves according to number of ganglia of origin

	1	2	3	4	5	6	7	Total
Greater								
Right	1	10	24	34	21	8	1	99
Left	4	8	41	24	17	7	0	101
Total	5	18	65	58	38	15	1	200
Bilaterality	0	0	4	4	2	2	0	12
Lesser								
Right	44	44	5					93
Left	56	37	5					98
Total	100	81	10					191
Bilaterality	23	12	0					35
Least								
Right	84	5						89
Left	91	5						96
Total	175	10						185
Bilaterality	77	2						79

table 1, bearing on the classification of the splanchnic nerves as based on the total number of ganglia of origin, and those summarized in table 2, indicating the specific ganglia from which these nerves arose, is explained by the fact that, whereas the greatest number of ganglia from which the greater splanchnic nerve, for example, was derived was seven, there was no single case in which this nerve arose from ganglia four to twelve inclusive. It can be seen from table 1 that there was only one example of a greater splanchnic nerve taking

origin from seven ganglia. In this single instance the nerve arose from the fourth to the tenth ganglia inclusively. Since it was brought out in table 1 that the highest percent of greater splanchnic nerves arose from three ganglia it is of interest to note that in this table the three ganglia from which the greatest percent of greater splanchnic nerves arose were seven, eight and nine (69.5%, 78% and 77.5%, respectively) as compared to the fifth, sixth and tenth ganglia from

TABLE 2
Origin of splanchnic nerves from specific thoracic ganglia

	4	5	6	7	8	9	10	11	12
Greater									
Right	5	23	60	72	79	81	61	10	1
Left	5	25	51	67	77	74	53	9	2
Total	10	48	111	139	156	155	114	19	3
%	5	24	55.5	69.5	78	77.5	57	9.5	1.5
Bilaterality	2	14	35	55	64	62	36	3	1
Lesser									
Right				1	1	11	42	75	23
Left						6	38	72	20
Total				1	1	17	80	147	43
%				.5	.5	8.5	40	73.5	21.5
Bilaterality						1	23	62	8
Least									
Right								8	86
Left								10	91
Total								18	177
%								9	88.5
Bilaterality								3	80

which only 24%, 55.5% and 57%, respectively, of the greater splanchnics were derived. As should be expected the fourth, eleventh and twelfth ganglia gave rise to the smallest percent of cases. The specific ganglia from which the highest percent of lesser splanchnics arose are shown in the table to be the tenth and eleventh, rather than the ninth and tenth. In 88.5% of the cases the least splanchnic nerve arose from the twelfth thoracic ganglion. It should be noted also that the greatest number of cases of bilateral symmetry of origin of these

nerves corresponds with the results outlined above with reference to the specific ganglia of origin. Thus the greater splanchnic nerve took origin bilaterally from the seventh, eighth and ninth thoracic ganglia in a greater percent of bodies than from the fifth, sixth and tenth ganglia; the lesser splanchnic arose bilaterally from the tenth and eleventh ganglia more frequently than from the ninth, there being only one body in which this nerve arose bilaterally from the latter ganglion, and the least splanchnic nerve arose bilaterally from the twelfth thoracic ganglion in 80% of the bodies. In regard to frequency of origin of the splanchnic nerves from specific thoracic ganglia according to right and left sides of the body the results are variable, although the bulk of the

TABLE 3
Normal splanchnic nerves

NERVE	NUMBER			"NORMAL" 200—100		BILATERALITY 100
	R.	L.	T.			
Greater	100	100	200	32	23	9
Lesser	93	98	191	15	14	1
Least	89	96	185	176	92	77
Total	282	294	576	223	129	87

evidence tends to indicate a greater frequency on the right side than on the left.

In table 3 is summarized the number of so-called normal splanchnic nerves found in this series of bodies. In the case of the greater splanchnic nerve thirty-two out of a total of two hundred nerves took origin according to the typical textbook description. However, normal greater splanchnic nerves were found in only 23% of the bodies examined. Still more impressive perhaps is the fact that normal greater splanchnic nerves were found on both sides of the body in only 9%. Only 15 normal out of a total of 191 lesser splanchnic nerves were found and these were present in only 14% of the bodies examined with a single case (1%) of bilateral symmetry. A total of 176 normal least splanchnic nerves were found in 92% of the bodies, 77% of which showed bilateral symmetry.

These results were arrived at by intentionally employing flexible or liberal criteria for normalcy. The method adopted for determining a normal splanchnic nerve was to allow arbitrarily some freedom of variation in origin from a sequence of ganglia. Thus in the case of the greater splanchnic nerve it was considered normal if it arose from ganglia 5 to 9 or 5 to 10 allowing for an absence of a branch of origin from one ganglion e.g. 5, 6, 8, 9, 10. On the other hand if a greater splanchnic nerve arose from ganglia 5, 8, 9, 10 or from 4, 5, 7, 8, 10 it was not classified as normal. As a matter of fact if the text-book description had been adhered to rigidly the following results would have been obtained: total number of normal greater splanchnic nerves out of 200 present was 18 (7 from ganglia 5, 6, 7, 8, 9 and 11 from 5, 6, 7, 8, 9, 10); total number of normal lesser splanchnic nerves out of 191 present was 11 (7 from ganglia 9, 10 and 4 from 9, 10, 11) and 165 normal out of 185 least splanchnic nerves (from twelfth ganglion). Still more striking is the small number of cases of bilaterality of origin of normal splanchnic nerves if the text-book description had been adhered to as a criterion. On this basis only 3% of the bodies had normal bilateral greater splanchnic nerves; 1% lesser splanchnics and 77% normal bilateral least splanchnics.

It should be added that in only four cases of apparent absence of the least splanchnic nerves did the lesser splanchnic nerve receive a branch of origin from the twelfth ganglion thus indicating, as Cunningham ('37) points out, that "it may be a branch of the lesser splanchnic nerve."

In regard to frequency of occurrence of the three types of splanchnic nerves in this series of bodies it can be seen from table 3 that the greater splanchnic was found in 100% of the bodies, the lesser splanchnic in 95.5% and the least splanchnic in 92.5%. In 2% of the bodies both lesser and least splanchnics were symmetrically absent while in 1% both were asymmetrically absent. These are the only instances found where both nerves were absent in the same body.

It should be pointed out that no data concerning double branches of origin from any one ganglion are recorded in the tables, since the objective of this study was to determine specific ganglia from which the nerves arose grossly. The question arose as to the method of recording gross origin of these nerves in those cases where branches arose from the sympathetic trunk between ganglia. In the relatively few cases in which careful dissection failed to demonstrate the branch of origin arising from the preceding ganglion, the origin was nevertheless recorded as such.

In view of the comparatively small amount of data presented in this paper the authors naturally hesitate to draw any dogmatic conclusions with regard to the mode of origin of the splanchnic nerves in man. However, sufficient evidence is presented to emphasize the increasingly apparent fact that the term normal as used so easily in many anatomical textbook descriptions is greatly abused and that human anatomical variations or structural differences between individuals as well as between sides of the body in the same individual are neglected or not sufficiently emphasized.

Mainland ('38), in discussing the failure of anatomists to recognize or comprehend the significance of the consistent biological phenomenon of variation, claims that the neglect or lack of sufficient emphasis upon structural differences between individuals "may have arisen primarily in the desire or tendency of anatomists for simple dogmatic statements." If this be true the tendency is certainly unjustifiable. In the case of the splanchnic nerves, the results of this study offer the challenge that the standard human anatomy textbook description of the origin of these nerves be reconsidered from the standpoint of possible variability of patterns.

SUMMARY

The types of variation in origin and average pattern of formation of the splanchnic nerves have been reviewed from a study of 100 cadavers.

The greater splanchnic nerve was present in 100% of the bodies on both the right and left sides; the lesser in 93% on the right side and in 98% on the left side and the least in 89% on the right as compared to 96% on the left side. In 2% of the bodies the lesser and least splanchnics were absent bilaterally. The greater splanchnic nerve arose symmetrically in 12% of the bodies, the lesser in 35% and the least in 79%.

The greater splanchnic nerve varied in origin from the fourth to the twelfth thoracic sympathetic ganglia, the lesser from the seventh to the twelfth and the least from the eleventh and/or twelfth.

The most frequent origin of the greater splanchnic nerve was from the seventh, eighth and ninth ganglia; of the lesser from the tenth and eleventh ganglia and of the least from the twelfth ganglion.

On the basis of a liberal interpretation of normalcy, normal greater splanchnic nerves occurred in 23% of the bodies (9% symmetrical); lesser splanchnics in 14% (1% symmetrical) and the least in 92% (77% symmetrical). However, on the basis of a strict application of the typical text-book description of normalcy, only 18 (9%) "normal" greater splanchnic nerves were found (3% symmetrical); 11 (5.5%) lesser splanchnics (1% symmetrical) and 165 (82.5%) least splanchnics (77% symmetrical).

Results of this study emphasize the need for a more careful consideration in text-books of human anatomy of the variability of patterns of the splanchnic nerves.

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STUDIES ON THE REPRODUCTIVE SYSTEM OF THE ALLIGATOR

VI. FURTHER OBSERVATIONS ON HETEROSEXUAL STRUCTURES IN THE FEMALE ALLIGATOR

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ONE TEXT FIGURE AND THREE PLATES (TEN FIGURES)

INTRODUCTION

The occurrence in female reptiles of structures homologous to functional components of the male reproductive system has been noted by several authors. Leydig (1853; cited by Braun, 1877) first described the persistence, in varying degrees, of mesonephroi and mesonephric ducts in female specimens of *Lacerta* and *Anguis*; Braun (1877) confirmed these observations and, with Schoof (1888), extended them to several other lizard genera. The latter author described in addition a rete ovarii in the lizards *Gongylus* and *Chamaeleo*. The retention of the mesonephric duct was also observed by Braun (1877) in female representatives of five snake genera. A mesonephric duct alone (van Wijhe, 1881; van den Broek, '33) or both mesonephros and mesonephric duct (Evans, '39) have been found in female specimens of five genera of turtles.

Some details have been published on the occurrence of heterosexual structures in female crocodilians. Gadow (1887) reported a female *Alligator mississippiensis* of a total length of 29 cm. in which there was present, apparently bilaterally, a fully formed mesonephric duct extending from the anterior end of the parovarium (the latter is only mentioned) to the cloaca. The mesonephros and its duct have been described in more detail for alligators of total lengths up to 19 inches

(Forbes, '37, '38 a, b, '40). Attention has at the same time been called to the constantly occurring medullary rest of the ovary. The medullary rest comprises the posterior end of the female gonad and is characterized by a total lack of cortex and by the persistence of the primary or medullary sex cords, elsewhere in a state of regression, as structures resembling the developing sex cords of the testis.

Recently the author has had an opportunity to study the reproductive tracts of three female alligators of total lengths up to $38\frac{1}{2}$ inches. Since the size of these animals was considerably greater than that of any female alligator for which persistent heterosexual components of the reproductive system have been described, and since these components showed interesting developments as compared to the earlier stages previously reported, a brief account of the findings is presented.

MATERIAL AND METHODS

The material studied was obtained from the reproductive tracts of three female alligators of total lengths of $32\frac{1}{4}$, 36, and $38\frac{1}{2}$ inches and from the reproductive tract of a male alligator of 85 inches total length. All evidence indicated that the females were still immature and that the male was mature.

The material was removed at death and fixed in Bouin's fluid. Complete serial sections, cut at 10μ , were made from part or all of both ovaries, together with the corresponding level of the mesonephros and its duct, of each of the three female specimens. Serial sections, also cut at 10μ , were made from blocks of tissue taken at four different antero-posterior levels of the testis of the male. All sections were stained with Mayer's acid hemalum and eosin.

OBSERVATIONS

Macroscopically, the female gonads, separated from each other by the vertebral column and the root of the mesentery, were seen to lie on the ventral surfaces of the kidneys. The cortical (ventral) surface of the flat, elongated ovary pre-

sented throughout most of its antero-posterior extent a pebbled appearance, due to the presence of numerous developing follicles. However, the caudal one-sixth of the ovary, tapering to a rounded point at its posterior end, had a smooth surface and firm consistency readily distinguishable from the anterior portion of the gonad. This wedge-shaped ovarian extremity was the medullary rest (Forbes, '37, '38 a, '40); its histological appearance will be described in a subsequent section.

Second, there could be seen projecting from beneath the postero-lateral margin of each ovary a slim but macroscopically visible mesonephric duct. The duct, as it progressed caudally to the cloaca, at the same time moved laterally until it paralleled and lay close to the root of the mesosalpinx. The latter actually carried the mesonephric duct in approximately the posterior one-third of the duct's extent. Finally, both oviduct and mesonephric duct entered the cloacal wall at about the same antero-posterior level.

Both mesonephric ducts were present and complete in each of the three female specimens. The ducts had the same size and general macroscopic appearance as their homologous structures, the vasa deferentia, in the reproductive tracts of three male alligators, approximately 3 feet in length, with which the female specimens were compared. The only gross difference was that the male specimens of course lacked a mesosalpinx for the support of the posterior portion of the vas deferens.

The mesonephros lay dorsal to the lateral margin of the ovary and was concealed by the latter.

The histological appearance of the various structures which were studied will be described separately.

1. *The medullary rest.* The medullary rests of the three female alligators showed slightly different degrees of development. In the most immature of the three, the cords and tubules of the rest were tortuous, somewhat anastomotic, four to six cell layers in thickness, and composed of several types of epithelium (fig. 2). The type representing most of the medullary epithelium closely resembled the germinal epithe-

lial cells from which, in the embryo, the primary sex cords are derived (Forbes, '40). These medullary cells were irregularly rounded and possessed an abundant, moderately eosinophilic cytoplasm. The round, basophilic nucleus usually had a well-defined chromatin network. The cells were compactly arranged. A basement membrane could not be distinguished.

A second cell type, much less numerous and largely limited to the posterior half of the medullary rest, was scattered through the medullary cords and occasionally comprised the greater part of a cord. This cell type did not occur in the tubules; it had some resemblance to germ cells but a greater diameter than the latter, a very clear, chromophobic cytoplasm, and a markedly basophilic nucleus which was smaller than that of the germ cell. Identical cells were seen in the medullary strands underlying the cortex anterior to the medullary rest; these strands were the conspicuously shrunken remains of the primary sex cords of the ovary proper. It is believed that in both locations the clear cells represented a degeneration of the epithelial cells originally comprising the medullary cords. The clear cells were slightly larger than the original epithelial cells. Brode's ('28) description and illustration of the "fat-laden" cells of the rudimentary right ovary of the post-hatching chick agree closely with the appearance of the clear cells of the alligator's medullary rest. Brode interpreted the presence of the "fat-laden" cells as evidence of medullary cord degeneration.

The irregular spaces separating the medullary cords were in some cases interstices which had contained tissue fluid; more frequently a blood vessel or sinusoid intervened between the cords. In addition, many but not all of the cords contained definite lumina and hence are more properly called tubules (figs. 2, 6). The relation of lumen to tubule wall was clearly seen in occasional isolated tubules; in addition, the lumina were lined with a distinctive epithelium which represented an additional cell type in the medullary rest (fig. 7). This epithelium consisted of a single uninterrupted layer of squa-

mous or flattened cuboidal cells. Flattening of the cells may have been due to the pressure of fluid within the lumen. Tubules of the variety just described will, for the sake of convenience, be referred to as type I (fig. 1).

Ovogonia, present singly or occasionally in groups of three or four, were scattered in moderate numbers and random fashion through the tubules and cords. If a lumen was present, the germ cells lay external, but usually close, to the thin layer of epithelial cells investing the lumen (fig. 7).

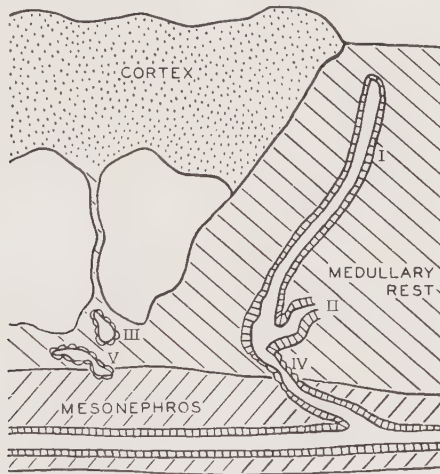


Fig. 1 Diagrammatic reconstruction of a longitudinal section through an ovary at the level of transition from cortex and medulla to medullary rest. The diagram indicates the relationships of the five types of gonadal tubules described in the text. A mesonephric tubule and the mesonephric duct appear below.

Germ cells were scanty in those limited areas where the clear cells were numerous. There was no indication of yolk formation within the germ cells; the latter were round and had a large, moderately basophilic nucleus and a rather clear cytoplasm (fig. 7).

The surface of the medullary rest was devoid of germinal epithelium. Instead, the rest was invested with a well-defined tunica albuginea; the free external surface of the latter was covered in turn with an uninterrupted single layer of typical

serosal cells (figs. 2, 4, 6). The tunica albuginea, composed of a compact, rather thin layer of connective tissue, completely surrounded the medullary rest except for part of the anterior boundary of the latter, where there occurred the sharply defined transition from tunic to typical ovarian cortex (figs. 1, 2). The tunic was thicker on the ventral than on the dorsal surface of the medullary rest.

In addition, many sections showed one or two septula arising from and extending approximately at right angles to the tunica albuginea and penetrating the medullary tissue for some distance (fig. 6). Most of the septula arose from the ventral portion of the tunica and, although thinner, were composed like the latter of dense connective tissue. The septula were repeatedly interrupted by the blood vessels and by medullary cords and tubules, and appeared to function as supporting structures. Finally, scattered strands of connective tissue fibers lay between many of the medullary cords and tubules and helped to form the stroma of the organ.

A second type of medullary tubule, in definite contrast to that previously described, occurred irregularly in approximately the dorsal one-third of the medullary rest and will be designated as type II (fig. 1). Such tubules were located in wedge-shaped areas which were often partly outlined by heavy connective tissue septula (figs. 5 and 6). The lumina of the tubules were lined with a simple low columnar or, less often, with a simple cuboidal epithelium, whereas the luminal epithelium of the tubules in the remainder of the medullary rest, as has been stated, was composed of much flatter cells. The tubules of the dorsal areas differed further in having a much smaller number of germ cells and in being more tortuous, more anastomotic, and of such an irregular diameter that they had almost a cavernous appearance. The dorsally located tubules appeared to be directly continuous with the type I tubules.

The medullary rests of the second female contained more ovogonia than the first, and the ovogonia occurred more frequently in groups (fig. 7). There was also a slight increase

in the clear cells of the medullary cords. Both the more numerous groups of ovogonia, indicative of additional mitoses, and the more numerous clear cells, suggestive of further regression of the posterior portion of the medullary rest, indicated that medullary rest development was more advanced in the second animal than in the first.

The medullary rests of the third female had developed even further. Whereas in the rests of the first two animals the ovogonia had laid in the walls of the (type I) medullary tubules (fig. 7), in the third female a considerable number of large aggregations of ovogonia occurred actually within the lumina of these tubules (fig. 4). Similar nests of germ cells were present in the true cortex (a derivative of the secondary sex cords) of the ovary where, however, well developed primary sex cords and tubules were lacking (fig. 2). It was quite apparent that in both cortex and medullary rest the large nests of germ cells were the result of repeated mitoses without subsequent migration of the daughter cells. Within the medullary rest, a section through a single nest often revealed 20 to 40, and sometimes more than 100, ovogonia. The medullary tubules containing the germ cell nests were more advanced than their fellows in that the squamous cells of the luminal epithelium usually rested on a delicate stratum of connective tissue fibers which were enclosed in turn by a slightly thicker wall of medullary cells of the usual variety. The tubules bearing the germ cell nests were limited to the medial half of the medullary rest.

In view of the total absence of cortex or germinal epithelium on the medullary rests of these and numerous other and younger female alligators (Forbes, '37 et seq.), it is evident that the development, just described, of the medullary rest tubules is a development not of aberrant cortical or secondary sex cords but of true medullary or primary sex cords.

Occasional short, discontinuous intra-medullary tubules, to be designated as type III, were also seen at the more anterior level of the ovary proper (fig. 1). These tubules, lined with a

simple squamous epithelium, usually occurred in or close to the ovarian hilus. Here, as has been previously pointed out (Forbes, '40), there is a small compact area formed by the relatively unregressed bases of the medullary cords. This area is situated at that point where the thin, widely separated, converging strands of connective tissue representing the degenerated portions of the medullary cords join each other just ventral to the mesonephros. Most of the intra-medullary tubules seen were within the hilus, but a few lay somewhat more peripherally and formed the bases of some of the medullary strands of connective tissue. Unlike the tubules in the medullary rest (types I and II), those in the hilus of the medulla proper were sometimes widely distended, apparently by fluid secreted into their blind lumina. Such distention had not proceeded to the stage of cyst formation. No tubules lined with cuboidal or columnar epithelium, similar to the type II tubules of the medullary rest, were seen in the medulla of the ovary proper.

2. *The rete system.* A rete ovarii was present in each of the three specimens, although poorly developed in the most immature of the three. The rete tubules¹ emerged from the dorsal side of the medullary rest as continuations of the medullary cords and tubules, passed obliquely through the dorsal tunica albuginea, and ended among the mesonephric tubules (figs. 1, 5, 6, 9, 10, 11). Instances of actual communication of rete tubules with mesonephric tubules occurred but were not numerous. It should be stated that the mesonephros, to be described in a subsequent section of this paper, lay immediately dorsal to the lateral half of the entire ovary.

The rete tubules, to be referred to as type IV, possessed lumina lined with a simple squamous epithelium resting directly on the dense connective tissue of the tunica which they penetrated. Like the specialized tubules (type II) of the

¹ The term rete tubule or rete canal will be used to designate those passages, lined with simple squamous epithelium, which lie dorsal to the medullary rest or medulla proper and extend from the medullary cords or tubules into the mesonephros.

dorsal areas of the medullary rest, the rete tubules were exceedingly tortuous and seemingly anastomotic and had lumina of such irregular contour that they were often quite cavernous in appearance.

Infrequent rete tubules, which will be referred to as type V because of their more anterior location, were observed at the level of the medulla proper, where they lay among but did not join the mesonephric tubules (fig. 1). The type V rete tubules appeared to be discontinuous; an individual tubule could not be traced without interruption from the hilus to the mesonephros. The anterior rete tubules, like those at the level of the medullary rest (type IV), were lined with a low simple squamous epithelium and were of irregular contour.

For the sake of comparison, an examination was also made of the rete system of an adult male alligator. The similarity of the rete testis of the adult and the rete ovarii (types IV and V) of the immature animals was striking; the only important difference was the considerably greater complexity and extent of the adult male system (compare fig. 8 with figs. 5, 6, 9, 10, 11). There can be no doubt of the homology of the male and female rete systems.

The classification of the various types of tubules thus far described was based on the location of the tubule and on the nature of the luminal epithelium. The five types may now conveniently be summarized as follows: Type I, all tubules of the medullary rest except those of the distinct, dorsally located variety; the luminal epithelium was simple squamous or simple flattened cuboidal. Type II, the distinct, dorsally located tubules of the medullary rest; the luminal epithelium was simple cuboidal or simple low columnar. Type III, the intra-medullary tubules at the more anterior level of the ovarian medulla proper; the luminal epithelium was simple squamous. Type IV, the rete canals at the level of the medullary rest; the luminal epithelium was flat simple squamous. Type V, the rete canals at the level of the ovarian medulla; the luminal epithelium was flat simple squamous.

These categories have been established for the sake of convenience; a detailed study of the histology of the adult female reproductive system may ultimately show them to be in part artificial.

3. *The mesonephros and its duct.* Vestigial mesonephroi were present in each of the three female specimens. The wolffian body, having failed to grow at the rate of the adjacent organs, if at all, was much less conspicuous than in younger animals. Glomeruli had entirely disappeared, but forty or more sections of renal tubules often appeared in a single section through anterior levels of the mesonephros. The smaller tubules were lined with a simple cuboidal epithelium; the larger tubules, with a simple low columnar epithelium (figs. 3, 5, 9, 10, 11). All of the tubules lay in a dense connective tissue stroma directly continuous with the dorsal portion of the tunica albuginea of the medullary rest. The relation of the rete system to the mesonephros has already been described.

Within the mesonephros, the wolffian duct could be recognized because of its relatively constant position in serial sections and because, in the posterior portion of the mesonephros, of the duct's greater diameter as compared to the mesonephric tubules (fig. 3). The duct was lined with a simple low columnar epithelium (fig. 3). The epithelial cells had round nuclei and a characteristic clear, chromophobic cytoplasm. The duct was encircled by a compact layer of circular smooth muscle fibers which lay centrally in close contact with the duct mucosa and intermingled peripherally with the dense connective tissue stroma of the mesonephros.

The vas deferens of the adult male was invested with a layer of circular smooth muscle very similar in its disposition to that seen around the wolffian duct of the female. The epididymal mucosa of the male was of the simple tall columnar type, while that of the vas deferens was a stratified columnar epithelium.

DISCUSSION

Precise data are not available on the age and size at which female alligators become sexually mature. Reese ('15, p. 17) expresses the belief that oviposition may begin at the age of 5 to 10 years; this would, on the basis of growth data supplied by various writers, be correlated normally with a total length of 6 feet or more. McIlhenny ('35, p. 64) states that, of over 175 female alligators which he examined during the breeding season, the smallest animal which contained eggs was 6 feet, 1 inch long. Hence it is apparent that the three female specimens described in this report were still definitely immature. Their age was judged to be about $2\frac{1}{2}$ years.

The observations presented in this paper in regard to the medullary rest and excurrent structures were essentially similar to those made in more than twenty younger normal female alligators (Forbes, '37 et seq.), with the exception that the three older animals showed somewhat more advanced development of the rete system and medullary rest.

It is pertinent to contrast briefly the development of the various heterosexual structures of the larger animals with those of the most advanced alligators (up to 19 months of age, 20 inches total length) for which descriptions have hitherto been available (Forbes, '37 et seq.). Within the medullary rests of the three older animals, the germ cells showed a marked increase in number, the medullary cords had sometimes undergone partial degeneration but had more frequently become tubules, the tunica albuginea and the stromal elements were markedly developed, and a rete system, present in the younger animals in an almost indistinguishable and very primitive form, was now clearly developed. The mesonephros and mesonephric duct had undergone some changes in that any remaining glomeruli had disappeared, smooth muscle elements had become conspicuous, and a slight increase in size of wolffian body and duct had probably occurred.

It is significant that in none of the females examined in this and preceding studies had the germ cells of the medullary

rests shown any tendency toward the accumulation of yolk, since it has been shown that macrolecithal eggs begin to appear within the ovarian cortex at some time between the third and sixth months after hatching (Forbes, '40). The appearance of clear cells in posterior areas of the medullary rests was an indication of some degree of regression; however, a much earlier and much greater degree of regression was seen in the typical medullary cords underlying the cortex. The latter had largely shrunk to thin connective tissue strands by the age of 6 months. Hence any tendency of the medullary rest to follow along the course of regression pursued by the medulla proper is slight at best during at least the first $2\frac{1}{2}$ years after hatching.

It would thus appear that the medullary rest, rete system, mesonephros, and mesonephric duct were making a definite, if retarded and somewhat erratic, attempt to develop along the lines followed at a much earlier age by the testis and homologous masculine accessory sex structures (see Forbes, '40, for details regarding the male). Due to the virtually complete lack of histological descriptions of the reproductive system of the adult female alligator, one can only speculate as to the further persistence of the female heterosexual structures and their condition at sexual maturity.

Schoof (1888) has described a limited rete system in female specimens of the lizard genera *Gongylus* and *Chamaeleo*. He states that one system of tubules, lined with cuboidal epithelium, begins within the ovary proper and, at the dorsal margin of the hilus, communicates with a second system of tubules; the latter, extending through an intervening band of connective tissue, reach and usually join the mesonephric tubules. The second tubule system thus corresponds to a rete system, and is so interpreted. Some of the tubules of the first, or intramedullary, system are said to end blindly at both their extremities and to have the appearance of cysts. No mention is made of intra-medullary tubules extending into the cortex or of any structure corresponding to a medullary rest.

The tubules of the first or intra-medullary system of the lizard, as described by Schoof, thus appear to be very similar to those tubules which, in the immature female alligator, occurred in the hilus of the medulla (type III) and perhaps also to the type II tubules of the dorsal portion of the medullary rest. It is possible that in the alligator the tubules of types II and III represented the first part of the rete system, but these tubules exhibited essential differences from the rete canals proper. The fact that the type II tubules were located in that portion of the medullary rest closest to the rete system and the mesonephros, however, does suggest that the tubules might have a potential efferent function. It also seems likely that the tubules of types II and III were serially homologous.

Schoof's description of what is obviously a rete system in the female lizard seems to be the only record in the literature of the occurrence of a rete ovarii in a post-hatching reptile. From his limited remarks, it appears that the rete ovarii of the lizard is essentially similar to that of the immature alligator.

The homology between the mesonephros and wolffian duct of the three female alligators and the epididymis and vas deferens of the male does not require emphasis.

In addition to the reptilian homologies which have been discussed, a most interesting homology appears to exist between the bilateral medullary rests of the alligator and the rudimentary right ovary of such birds as the hen. A comparison of Brode's ('28) detailed description of the right ovary of the sexually immature and mature hen (sexual maturity is said to be attained at the age of 6 months) with the present observations on the medullary rest reveals many points of striking similarity. In the hen, according to Brode, the right ovary is largely or entirely composed of persistent medullary cords. In some cases, the cords contain a large number of "clear" or "fat-laden" cells. In other cases, the cords have developed distended lumina of irregular contour; the walls of such tubules are indefinite, and their mucosal

cells are flattened. The surface of the adult right ovary is said to be covered with "a thick layer of connective tissue continuous with the adventitious layers of the post-caval vein" (p. 34); such an investment certainly corresponds to the tunica albuginea of the alligator.

In other respects, the right ovary of the adult hen shows further regression than the medullary rest of the immature alligator. Primordial germ cells have disappeared, with rare exceptions, by the end of the third week after hatching. Many of the medullary cords are shrunken, and the ovary, invaded by a large amount of connective tissue, has a loose, reticular appearance. A similar but less developed stroma in the alligator's medullary rest has been interpreted by the writer as further evidence of its testis-like nature. Finally, germinal epithelium in varying degrees of development was present on the rudimentary right gonads of four of the twelve hens aged 3 weeks to 6 months whose ovaries were sectioned and, apparently, on not more than four of the right ovaries of the fifteen specimens aged 18 months which were examined. In the alligator, a germinal epithelium is present over the entire ovary, including the medullary rest, in embryonic stages, but all traces of germinal epithelium have disappeared from the medullary rest by the time of hatching (Forbes, '40).

Brode also found that definite remnants of both the right mesonephros and its duct persist even in the 18-month-old hen, and believed that the left mesonephros and duct similarly persist. In the adult fowl the right mesonephros was said to consist of convoluted tubules and sometimes to show a degree of involution. Such a description applies to both mesonephroi of the female alligator. The right wolffian body of the adult hen, according to Brode, is somewhat removed from the right ovary but is joined to the latter by long rete canals. The hen's rete tubules are poorly defined at 3 weeks after hatching but are very conspicuous in the adult. Similarly, in the alligator the rete tubules are difficult to distinguish in stages as late as 18 months after hatching but, as has been indicated,

are quite obvious at an estimated age of $2\frac{1}{2}$ years. On the basis of Brode's data it cannot be determined whether the rete canals linking the right ovary to the wolffian body in the mature hen are longer than the bilateral rete tubules of the immature female alligator. Brode's findings, then, for the rete system, mesonephros, and mesonephric duct of the mature hen, and his interpretation that these structures constitute a complete excurrent system for the right ovary, are in essential agreement with the author's observations and interpretation of the homologous structures in the female alligator.

Thus such differences as exist between the heterosexual structures discussed for the hen and the parallel structures of the immature female alligator, aside from laterality, are differences of degree or of differential rates of development. That these differences should exist is scarcely surprising, particularly when one recalls that the maturation of the hen's reproductive system, accomplished at 6 months of age according to Brode, is relatively very rapid as compared to the attainment of sexual maturity in the alligator at an estimated age of 6 years or more. In addition, the fact that the animals in question are representatives of different vertebrate classes should account for a degree of variation.

Embryological studies of the reproductive systems of various birds and reptiles have established the fact that gonads, rete, mesonephroi, and gonoducts present a remarkably bisexual appearance during one phase of pre-hatching development. Thus a developmental basis is established for the post-hatching retention of heterosexual components of the reproductive system, a phenomenon which has been shown to occur to a striking degree in several reptilian and avian forms. The female alligator and the hen offer conspicuous examples of this phenomenon.

Finally, in the light of phylogenetic relationship, it is most interesting that a markedly bisexual condition should persist in female representatives of both the reptilian and avian classes, and that in the hen the masculine complex occurs chiefly unilaterally and includes all or nearly all of one gonad,

while in the female alligator the corresponding elements are present bilaterally, chiefly at the posterior levels of both gonads.

SUMMARY

The persistence and development of the medullary rest, the rete system, and the mesonephric tubules and duct in three immature female alligators of a total length of approximately 3 feet is described and discussed. The bilateral medullary rest comprises the posterior portion of the ovary and is characterized by persistent medullary cords which in some areas possess lumina and large numbers of germ cells, by a definitive, testis-like stroma and tunica albuginea, and by the total absence of germinal epithelium and cortex. The rete canals provide an excurrent pathway from the medullary rest to the mesonephric tubules, which in turn communicate with the mesonephric duct. The suggestive similarity of the medullary rest to the testis of a younger male and the striking homology of the excurrent system of the female with that of the male are pointed out.

It is further suggested that an interesting and important homology exists between the bilateral medullary rest and its potential excurrent system in the female alligator and the rudimentary right ovary and its potential excurrent system in the fowl; the evidence is discussed in some detail.

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PLATE 1

EXPLANATION OF FIGURES

2 The transition between ovarian cortex and true medulla (above and left) and medullary rest (below and right) is illustrated; this appearance was characteristic of all three female specimens. The dense tunica albuginea extends diagonally across the entire field and demarcates the medullary rest. A developing cortical ovum is seen at the left. Note the medullary tubules (type I) with their flat epithelial lining (below); these tubules are derivatives of medullary (primary sex) cords. The cords seen in the upper half of the picture are chiefly cortical (secondary sex) cords. $\times 120$.

3 A typical section through the persistent female mesonephros and mesonephric duct (left of center) at the level of the medullary rest. The glomeruli have disappeared, but the mesonephric tubules are still well defined. Smooth muscle fibers encircle the wolffian duct. $\times 100$.

4 A section of the ventral portion of the medullary rest in the female showing most advanced development. A well-formed medullary tubule (T), shaped somewhat like the numeral 3, is indicated in the center of the field by arrows. Note the large, ventrally located nests of germ cells (G) in cross sections of two of the tubules. Part of the tunica albuginea may be seen at upper right. Germinal epithelium is entirely absent. The body cavity lies to the right. $\times 100$.

5 Mesonephric tubules (M) appear at upper left; part of the dorsal portion of the medullary rest, at center and lower right. The specialized, dorsal medullary rest tubules (type II), lined with cuboidal and low columnar epithelium, appear at extreme lower right. Rete tubules (type IV), lined with simple squamous epithelium, are seen at center and below. $\times 100$.

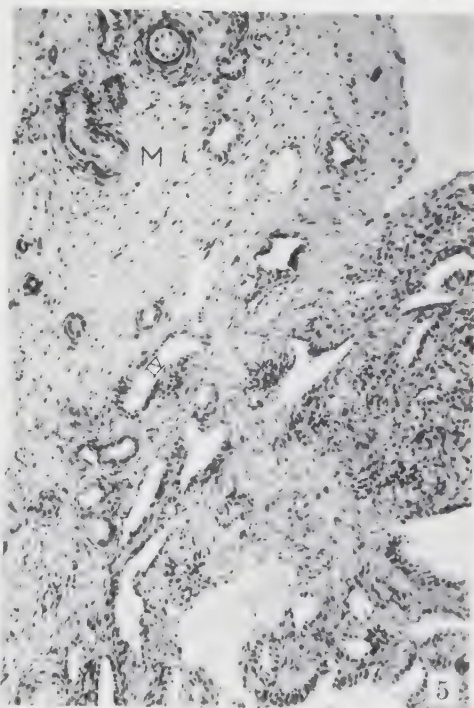
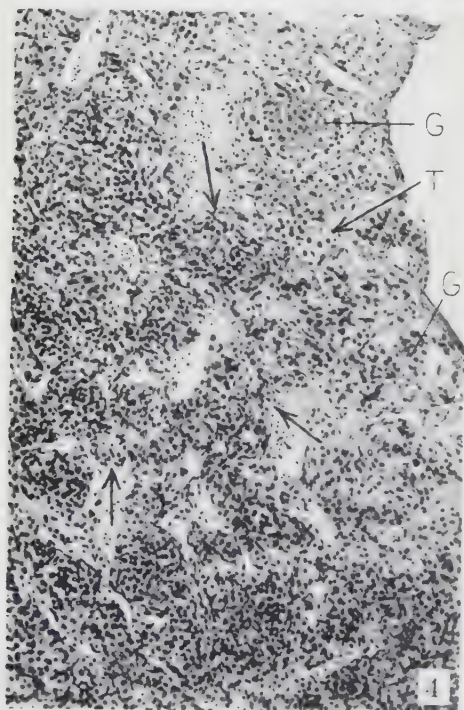
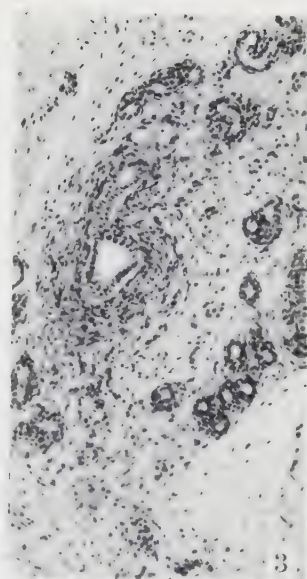
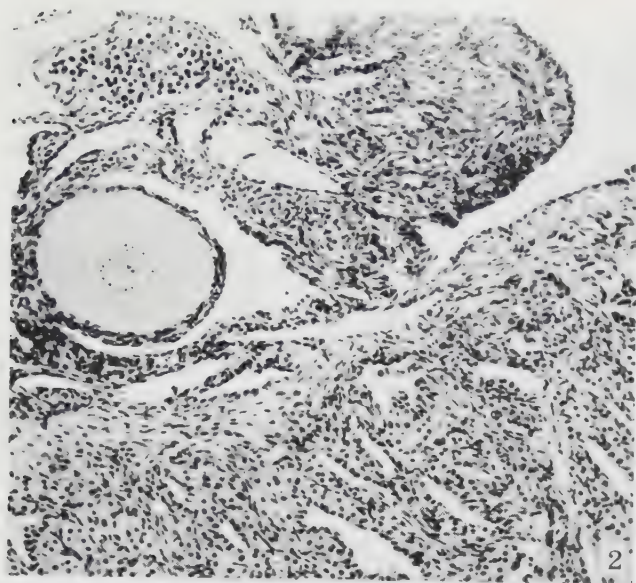


PLATE 2

EXPLANATION OF FIGURES

6 In this female the medullary rest, of which another section is seen in figure 2, showed slightly less advanced development than that seen in figure 4. A small portion of the tunica albuginea (ventral) is seen at extreme upper right. The connective tissue subjacent to the basal (dorsal) area of the rest appears at lower left. Most of the medullary rest is occupied by cords and (type I) tubules; the latter are lined with flat epithelial cells. Just below center, a type II tubule merges into a rete tubule (type IV); another of the latter is seen at extreme left. Part of a septulum is seen above and to the right of center. See figure 7 for part of this field at higher magnification. $\times 100$.

7 A more highly magnified view of part of the field (as indicated) seen in figure 5. Note the well-defined epithelial cells lining the lumina of the medullary (type I) tubules and the germ cells (G) lying beneath the luminal epithelial cells. $\times 500$.

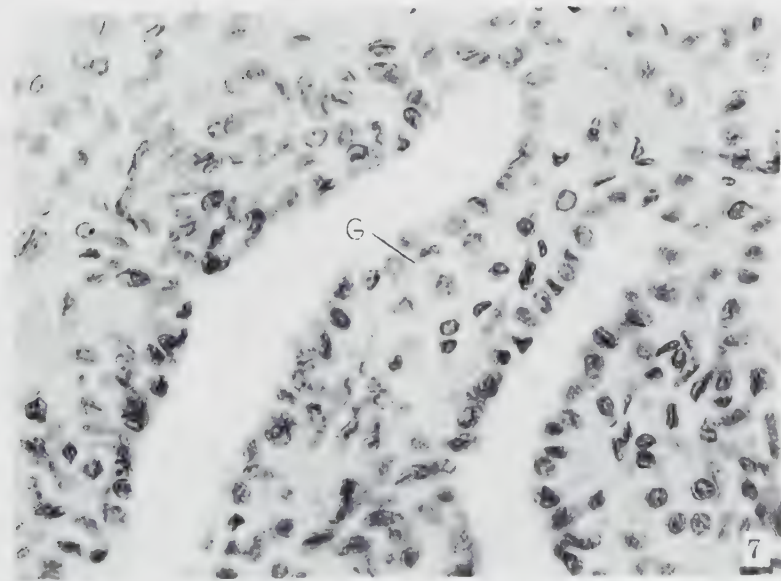
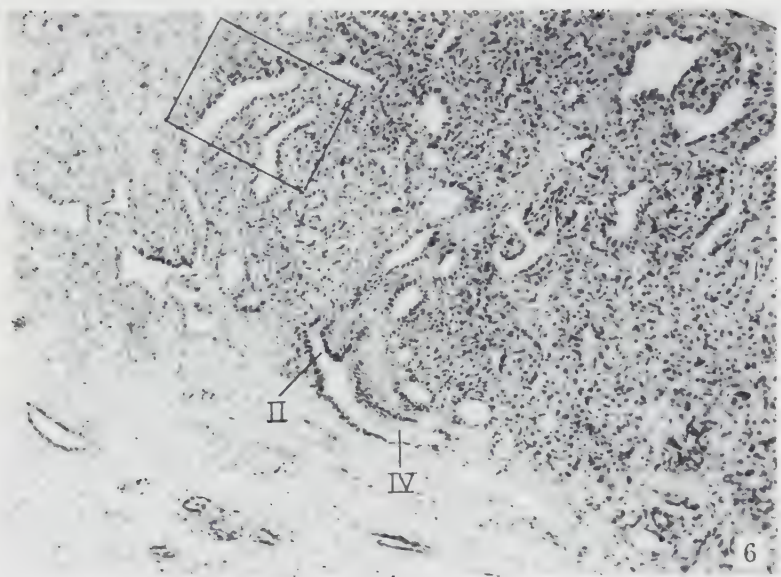


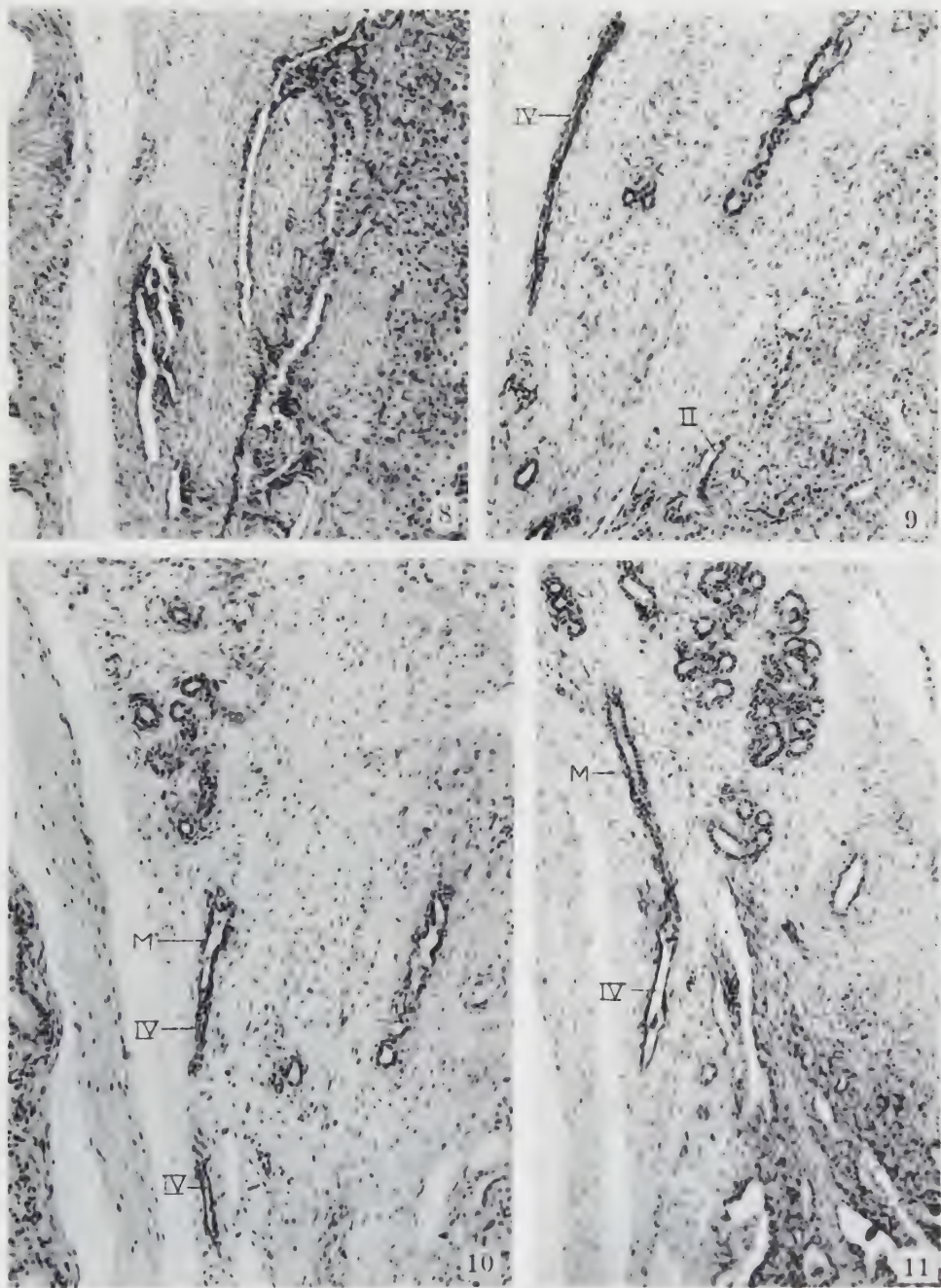
PLATE 3

EXPLANATION OF FIGURES

8 Typical rete tubules as seen in the adult male alligator. The tubules are tortuous and lined with simple squamous epithelium. The margin of the kidney lies at the left; that of the testis, at the right. $\times 100$.

9 and 10 The area between mesonephros and medullary rest as seen in two successive serial sections. The long rete tubule (type IV), seen at left in both views, ends in a mesonephric tubule (M). The dorsal margin of the medullary rest appears at lower right in figure 9; part of the mesonephros is seen above in figure 10. The ventral margin of the metanephros lies at the left in figure 10. Compare the rete tubules in figures 9 and 10 with those in figure 8. $\times 120$.

11 Another view of the female mesonephros (above), rete tubules of type IV (center), and dorsal medullary tubules of type II (below). Note the junction of rete (IV) and mesonephric tubules (M) and the characteristic epithelial lining of each. Compare these rete tubules with those in figure 8. $\times 100$.



AXON REACTION IN MOTOR NEURONS AND ITS EFFECT UPON THE END-BULBS OF HELD-AUERBACH

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TWO FIGURES

The effect of axon injury upon the parent nerve cell body has been the subject of many careful studies. Characteristic alterations in cell shape and size, nuclear position, shape and size and changes in the various cytoplasmic constituents have all been described by many authors. The question of changes in the end-bulbs of Held-Auerbach or boutons terminaux on such reacting cells has received less attention. Lawrentjew ('34) failed to observe any alterations in the end-bulbs on cells undergoing retrograde or axon degeneration in sympathetic ganglia. Barnard ('38) reported the disappearance of end-bulbs on ventral horn cells of the cat's spinal cord following ventral root section. The present author has reported a decrease in the number and size of end-bulbs under similar conditions ('39 a); but Schadewald failed to see any change in the nucleus of the fourth and sixth cranial nerves after removal of the orbital contents of cats ('40).

Axon terminations, in the form of end-bulbs of Held-Auerbach or boutons terminaux, are present in large numbers on the cell body, dendrites and proximal portion of the axon of neurons in the ventral horn of the cat's spinal cord. In the present series of experiments retrograde "degeneration" or axon reaction was produced in such cells by peripheral nerve injury. The end-bulbs were studied quantitatively to determine any alteration in their number or size on such reacting cells.

MATERIAL AND METHODS

Peripheral nerve lesions were produced in nine healthy, mature cats in the following manner. The right sciatic and femoral nerves were exposed in the thigh, using ether anesthesia and strict aseptic precautions. These nerves were then ligated, sectioned immediately distal to the ligature and the central stump buried by suturing into an adjacent muscle. Traction on the exposed nerves was avoided.

The animals were sacrificed at the end of 7, 10, 14, 20, 26, 34, 105 and 120 days. With the animal anesthetized with ether the spinal cord in each case was perfused through the vascular system with 15% neutral formalin. Blocks were removed from the lumbo-sacral enlargement of the cord and sections prepared by the following histological methods:

(a) Cajal's reduced silver nitrate method, modification 4. This technique yields well stained sections demonstrating the end-bulbs of Held-Auerbach (Barr, '39 b).

(b) Toluidin blue, for Nissl substance.

(c) Bodian's modification of the protargol method, for neurofibrils (Bodian, '36).

All sections were cut 10 μ in thickness from blocks imbedded in paraffin.

OBSERVATIONS

Cell changes characteristic of axon reaction were seen in the dorso-lateral group of the right ventral horn in all animals. In these cells the shape and size of the cell body, the position, shape and size of the nucleus and the appearance of the Nissl substance and neurofibrils were observed and compared with the normal control cells in the dorso-lateral group of the opposite side. After establishing the degree of axon reaction in the affected cells the number and size of end-bulbs on cells of the dorso-lateral groups of the right and left ventral horns were determined.

Effect of axon section on cell body and nucleus

The changes in the cell body and nucleus which follow axon injury are well known and will be described here only in

sufficient detail to indicate the degree of axon reaction encountered in the present series of experiments.

(a) Swelling of the cell body. An increase in the cross sectional area of the cell body of approximately 20% in animals surviving for periods of 7, 10, 14, 20, 26 and 34 days and 7% in animals surviving for periods of 68, 105 and 120 days is a characteristic feature of the cells of the dorso-lateral group on the operated side. Pallor of the cytoplasm, especially in the peri-nuclear region, is constant in these swollen, reacting cells stained by the reduced silver method.

(b) Position, shape and size of the nucleus. The nucleus of the normal neuron of the dorso-lateral group is situated centrally or nearly so in the cell body and is round or slightly oval in cross section. In the cells reacting to axon injury the nucleus migrates toward the cell membrane and returns to a more central position as the neuron recovers from the injury (after 105 and 120 days). Similarly, the cross-sectional shape of the nucleus becomes more generally oval in reacting cells and the normal shape is restored as the nucleus returns to its central position in the cell body.

During the early stages of axon reaction (7 and 10 days) there is little alteration in nuclear size. In later stages (14 days and over) nuclear shrinkage is a constant feature and amounts to approximately 13% of the cross-sectional area of the nucleus of the normal side.

There is no apparent alteration in the general appearance, position, shape or size of the nucleolus in reacting cells.

(c) Nissl substance. The Nissl substance is reduced in quantity and the large angular Nissl bodies are broken down into fine, dust-like particles in reacting cells. Nissl bodies of normal shape, size and density are restored during the recovery period (120 days).

(d) Neurofibrils. In sections stained by Bodian's modification of the protargol method, neurofibrils in the normal cell are arranged in bundles passing through the cell body. Unstained, irregular areas, corresponding in size and shape with the Nissl bodies of toluidin blue stained cells, appear between

the neurofibrillar bundles. During the various stages of axon reaction the bundles of neurofibrils become dispersed and indistinct. Restoration of the normal arrangement of neurofibrils occurs as the cells recover from axon injury (120 days).

The dendrites do not take part in axon reaction of a degree encountered in this series.

Effect of axon section on end-bulbs of Held-Auerbach

When satisfied that axon reaction of a moderately advanced degree was present in the dorso-lateral group of cells on the operated side evidence of changes in the end-bulbs of Held-Auerbach was sought in the following manner. The number

TABLE 1

SURVIVAL TIME (DAYS)	AVERAGE NUMBER OF END- BULBS PER CELL SECTION		NUMBER OF END- BULBS ON REACTING CELLS AS PERCENTAGE (CONTROL 100%)	AVERAGE SIZE OF END BULBS (μ)	
	Control cells	Reacting cells		Control cells	Reacting cells
7	68	64	94	0.8×1.4	0.8×1.4
10	31	28	90	1.0×1.6	0.9×1.5
14	36	34	94	1.1×1.8	1.1×1.8
20	33	34	103	1.0×1.8	1.0×1.6
26	50	47	94	1.0×1.5	0.9×1.5
34	53	48	91	0.9×1.5	0.8×1.3
68	43	41	95	0.9×1.5	1.0×1.5
105	33	33	100	0.9×1.5	0.8×1.3
120	40	36	90	0.9×1.5	0.8×1.4

of end-bulbs on cell body and dendrites of 100 nucleoli containing cell sections in each of the right and left dorso-lateral groups was counted, using the material prepared by Cajal's reduced silver nitrate method. From these counts an average figure was obtained for cell sections of the dorso-lateral groups of the control and operated sides for each animal.

As an aid in detecting alterations in end-bulb size fifty end-bulbs on cell bodies of normal and reacting neurons were measured and average figures obtained.

The results of end-bulb counts and measurements are presented in table 1.

The average figures for numbers of end-bulbs per 10μ cell section are indicative of the results which may be expected in sections of the cat's spinal cord stained by modification 4 of Cajal's reduced silver nitrate technique. Due to technical difficulties the completeness of staining of these minute nerve structures varies from animal to animal and even from cell to



Fig. 1 Drawing of a normal motor nerve cell of the dorso-lateral group of the ventral horn on the control side.

Fig. 2 Drawing of motor nerve cell of the dorso-lateral group of the ventral horn 26 days following peripheral nerve section. The number and appearance of the end-bulbs of Held-Auerbach do not differ materially from that on the normal cell illustrated in figure 1.

Drawings prepared by Margaret Corrin from sections stained by modification 4 of Cajal's reduced silver nitrate technique. Magnification approximately $\times 900$.

cell in the same section. Counts of 150 to 180 end-bulbs per cell section on cells of both control and operated sides of the cord are frequently encountered. These higher values represent the condition in the living cord more accurately than the average figures given in the table.

In the numerical studies counts were made on control and reacting cells in the same sections. These cells had been subjected to identical physical and chemical conditions during the

histological preparation of the tissue. The counts on experimental cells, therefore, may be considered to be adequately controlled in spite of the apparently inevitable variation in staining.

As shown in the table there is a tendency toward fewer stainable end-bulbs on motor cells undergoing axon reaction than on normal cells of the same group in the contra-lateral side of the spinal cord. This decrease in stainable end-bulbs was absent in the 20 and 105 day cats and did not exceed 10% of the average number on normal cells in any animal of the present series. The lower counts generally recorded on reacting cells is due to a slight decrease in the number of stainable end-bulbs on the cell bodies of such neurons. Neither the dendrites nor the end-bulbs on dendrites take part in the reaction to peripheral nerve injury.

There is a tendency, as determined by general inspection (figs. 1 and 2) toward a slight decrease in the size of end-bulbs on the cell bodies of reacting neurons. This decrease in size is inconstant and extremely small as shown by the average figures for end-bulb size recorded in table 1.

No alteration in the intrinsic structure of individual end-bulbs or in their relationship to the cell surface could be detected on cells undergoing axon reaction.

DISCUSSION

The slight decrease in the number of end-bulbs visible on reacting cells need not be accepted as an actual decrease in the number of end-bulbs on such cells in the living state. The delicate and uncertain nature of silver staining methods is well known. Altered physico-chemical conditions in the region of neurons undergoing axon reaction may influence the staining affinity of end-bulbs on such neurons and account for the somewhat lower counts generally obtained. The normal appearance of individual end-bulbs on reacting cells supports the view that the number of end-bulbs on such cells in the living state does not depart from the normal.

The decrease in the size of end-bulbs on reacting cells was extremely small. However, end-bulbs on the normal motor cells of the dorso-lateral group of the ventral horn are normally the smallest of the spinal cord. Possibly axon reaction in cells of the medial groups of the ventral horn on which the end-bulbs are normally of larger size might produce more readily demonstrable end-bulb shrinkage. In this connection it is interesting to note that the nuclei of reacting cells are almost uniformly smaller than those of normal cells of the same group. The decrease in nuclear volume and such decrease in end-bulb size as does occur may be due to the transfer of water through nuclear and cell membranes into the cytoplasm of the swelling, reacting cell body.

In spite of the limitations of the staining technique the observations recorded here clearly indicate that end-bulbs are relatively inert to axon reaction. The slight changes noticed in the end-bulbs is in contrast with the marked response of the cell body, nucleus and cytoplasmic constituents to axon injury. The absence of any extensive changes in the end-bulbs supports the view of neurofibrillar discontinuity at the synapse and the theory of anatomical unity of the neuron.

SUMMARY

1. Axon reaction was produced unilaterally in cells of the dorso-lateral group of the ventral horn in the spinal cords of nine cats.

2. The number of end-bulbs visible on such nerve cells, stained by the reduced silver nitrate method of Cajal, was not less than 90% of the number on normal cells of the same group in the contra-lateral side of the cord at any stage of axon reaction observed. The decrease in stainable end-bulbs on reacting cells is interpreted as due to technical difficulties rather than an actual decrease on such cells in the living state.

3. The end-bulbs on reacting cells are normal in appearance except for very slight decrease in size of end-bulbs on the cell body.

4. The absence of significant changes in the end-bulb in the presence of axon reaction compared with the marked changes in the cell body, nucleus and some cytoplasmic constituents does not support the view of neurofibrillar continuity at the synapse.

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THE EMBRYOLOGY AND POSTNATAL DEVELOPMENT OF THE PROSTATE GLAND IN THE FEMALE RAT ¹

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THREE TEXT FIGURES AND TWO PLATES (EIGHT FIGURES)

In 1938, Witschi, Mahoney and Riley published some data on the frequency of occurrence, the morphology, and the hormonal responses of the female prostate in several strains of rats. Their paper also contains a review of the older literature on this subject. Later, a short account of the effect of selective breeding on the frequency of the gland was also made (Witschi and Riley, '39), emphasizing the hereditary nature of strains with high and low frequencies (so-called "prostate" and "non-prostate" strains).

Price ('39) reported on the gland as it appeared in nineteen female rats ranging in age from 5 days to adults. She observed the regression of the prostate in females over 40 days of age, and compared it to the involution seen in the prostate gland of young castrated male rats. The possibility that the adrenal glands may maintain the prostate of the castrated male and that of the normal female in a functional state in young animals was drawn into consideration by this author.

Recently, Lehmann ('38) reported paired "paravaginal glands" in mature female *Hemicentetes*. In position and structure they resemble female prostate glands, but she finds they

¹ Aided by grants from the National Research Council, Committee for Research in Problems of Sex; grants administered by Professor Emil Witschi.

have no orifices, either in the vagina or in the urethra. The embryological development of the glands was not studied.

Since the work of Price covers only the post-natal period and is based on rather haphazardly encountered specimens with prostates, it seemed worthwhile to enlarge on it by making a comparative study of our prostate and non-prostate strains. This study of normal development, on which a short abstract (Mahoney, '39) was recently published, suggested itself in connection with our experiments on the effects of pre- and post-natally administered sex hormones.

I wish to express my gratitude to Professor Emil Witschi for suggesting this problem and for his helpful advice and criticism during the progress of the investigation.

MATERIAL AND METHODS

Albino rats of two strains were used in this study. The first, designated hereafter as the prostate strain (P.S.) was derived by selective breeding from Wistar Stock and showed a 93.0% incidence of the gland in the females. The second one, the non-prostate strain (N.P.S.) was derived from animals of the Breeding and Laboratory Institute Stock (New York, N. Y.). Over 400 laparotomies and postmortem examinations show that N.P.S. females over 13 days of age have neither rudiments nor vestiges of prostate glands.

The embryos for this study were preserved at 12-hour intervals, and embryonic age was dated from the time of observed copulation.

The urogenital tracts of forty-two female and eleven male embryos were serially sectioned. Of these, thirty-eight were animals of the prostate strain, and fifteen were of the non-prostate group.

One hundred and seven normal female rats were used in the investigation of the post-natal development of the gland. The entire prostate areas of sixty-three of these females were serially sectioned.

EMBRYONIC DEVELOPMENT

A. Male

The embryology of the prostate gland in the male rat has been described by Price ('36), but for comparison with that of the female a brief description will be given here. The primordia of the male prostate gland first appear in rat embryos of the age of 19 days 8 hours. The urogenital sinus gives rise to solid buds that are to form the tubules of the various lobes of the prostate gland. Ventral to the capsule of the sinus, at the site of the future ventral lobes, appears a horseshoe-shaped pad of condensed stroma which later will form the intertubular stroma of these lobes of the gland. In later embryonic life this pad becomes indistinct and merges with the stroma around the sinus. The stroma at the site of the dorsolateral lobes and the coagulating gland is never so clearly demarked.

During embryonic life the epithelial buds from the urogenital sinus grow in length, coil, branch, and increase in number, but are still solid cords of cells at birth. Lumen formation in early postnatal life begins in the ducts and progresses distally in the tubules of the gland.

B. Females of the prostate strain

Nineteen day 8 hour embryo. In females as in the males, the first rudiments of the prostate are found in embryos of 19 days 8 hours development. The gland originates similarly from two primordia. One of these is a condensed pad of stroma lying ventral to the urethra, caudal to the neck of the bladder, at the site of the future prostate gland. Its further development during embryonic life consists merely in a slight increase in size.

The second primordium, which arises caudal to the stromal pad, consists of one, two, or more (see table 1) cords of cells on each side budding from the urethral epithelium and projecting ventrolaterally into the surrounding mesenchyme (fig. 4). If there are two or more cords the buds are distributed over a length of the urethra varying from 100 to 150 μ . These

cords are to form the future glandular tubules and the efferent ducts of the prostate.

These epithelial buds differ in length, the shortest being 30 μ long, the longest 70 μ . They are usually uncurved and somewhat longer on the right side than on the left.

The cells of the cords are compactly arranged with relatively small amounts of deep-staining cytoplasm, and irregular round or oval nuclei. They resemble closely the peripheral cells in the urethral epithelium from which they arise, and retain this appearance until late in embryonic life.

TABLE 1

Epithelial buds in female embryos of the prostate strain

AGE (DAYS)	NO. OF RIGHT PROS- TATE BUDS	TOTAL LENGTH (μ)						NO. OF LEFT PROS- TATE BUDS	TOTAL LENGTH (μ)		
		R ₁	R ₂	R ₃	R ₄	R ₅	R ₆		L ₁	L ₂	L ₃
19 $\frac{1}{3}$	2	49	33					2	30	52	
19 $\frac{1}{3}$	2	169	85					1	39		
19 $\frac{1}{2}$	2	39	85					3	29	50	53
20	3	69	102	72				2	66	82	
20	3	59	62	86				2	29	135	
20 $\frac{1}{2}$	3	144	89	15				2	52	184	
20 $\frac{1}{2}$	3	46	182	108				2	42	118	
21	3	67	154	79				2	53	163	
21	2	149	148					1	194		
21 $\frac{1}{2}$	2	199	125					2	159	248	
21 $\frac{1}{2}$	5	48	36	184	258	59		2	212	176	
22	3	225	116	46				0			
22	6	216	356	59	105	6	195	3	89	229	262

In the following description of the further embryonic development of the gland, reference will be made only to the epithelial buds (fig. 1). The stromal pad does not differ in following stages from its appearance in the 19 day 8 hour embryo.

Twenty day embryo. In the 20 day embryo there is a marked tendency for the epithelial cords to bend either caudally or cranially and run parallel to the urethra for distances of from 10 to 30 μ . The buds extend out from the urethra to the point

of curvature for distances varying from 10 to 100 μ in different embryos.

A second feature of the 20 day embryo is the relative increase in length of the second bud over that of the first (cranial) bud. The average length of the cranial cords is 43 μ , while the second cords have an average length of 81 μ . A third pair of

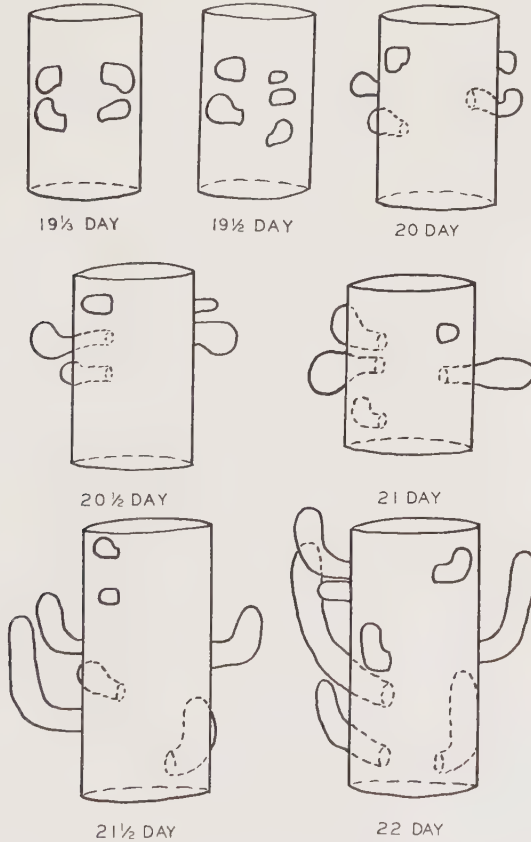


Fig. 1 Diagram showing budding of epithelial cords from the urethra in female embryos of prostate strain, viewed from ventral side. Relative proportions determined by camera lucida reconstructions. Note that early buds arise ventrally, while buds in later stages arise laterally or from dorso-lateral aspect of urethra. Note also increased length of buds with age, cranial course assumed, and increase in bud number in older embryos. $\times 260$.

buds, or a third unilateral bud is often seen in embryos of this stage.

Twenty day 12 hour embryo. At the 20 day 12 hour stage there is further increase in length of the cords, and they now tend to parallel the urethra for longer distances. The majority of the buds now grow cranially, running, in some cases, as far as 90 μ forward. The most usual bud number remains three on the right and two on the left, and the third cord (caudal) now often shows a slight cranial or caudal curvature.

In embryos of this stage the cords often twist as they grow out through the mesenchyme. As many as three bends are often noted in a single cord.

Twenty-one day embryo. This stage is marked chiefly by further lengthening of the buds. The distal ends of a few of the cords now lie at, or almost at, the periphery of the urethral mesenchyme, where the urethro-vaginal sheath is being formed.

The cells of the cords are now more markedly organized into a compact peripheral layer, and a more disperse central group of cells. However, as yet there is no evidence of a lumen in the cord, and all the cells retain their dark-staining cytoplasm.

Twenty-one day 12 hour embryo. Continued elongation of the cords characterizes this stage. Many measure over 100 μ , and some attain a length of 160 to 180 μ . There is evidence of a tendency to further increase the number of buds as embryonic life proceeds, and four or five buds are often found on the right side. In contrast, the left side of the urethra never develops more than three buds.

The cells in the cord when seen in cross section now begin to show some differentiation. The peripheral layer remains compact, and deep-staining, but the cytoplasm of the cells toward the center of the cord begins to take on a lighter appearance.

Later embryological development. No further changes in the development of the female prostate gland occur during embryonic stages after the 21 day 12 hour stage. There is some continued lengthening of the epithelial cords, but otherwise the primordia both remain unaltered in appearance.

C. Females of the non-prostate strain

Only one of the two primordia of the prostate gland is a constant feature of N.P.S. female embryos. This is the stromal pad, ventral to the urethra. Epithelial buds from the urethra, are less often observed. Out of twelve embryos examined, only five show one or two buds, and in only one is the budding bilateral. No embryo shows more than a single bud on one side.

The epithelial buds that do appear are small in comparison with those of the P.S. female embryos. Of the total of six found (five right, one left) only two turn cranially. Histologically, both the epithelial buds and the ventral stromal pad are similar to those in females of the prostate strain.

POST-NATAL DEVELOPMENT

A. Macroscopic

The prostatic lobes of the newborn female rat are paired structures located ventrolaterally to the urethra just caudal to the neck of the urinary bladder. The two lobes are joined by a bridge which is usually somewhat thinner than the lobes themselves. This connecting bridge, however, may be of sufficient thickness to cause the structure to appear like a single lobe lying closely applied to the ventral surface of the urethra, and extending a short distance dorsally on each side (fig. 2).

The newborn N.P.S. female rat possesses a thickened pad on the ventral surface of the urethra caudal to the neck of the bladder (fig. 2). This pad cannot be distinguished from the prostate of the newborn P.S. female.

As growth continues in both strains the connecting bridge becomes thinner and the lobes become more distinct. By the ninth day of life this bridge has disappeared. The exact position of the lobes varies in different animals, and frequently the medial edge of the lobe of one side lies in the ventral mid-line or even extends to the opposite side. In the N.P.S. females the lobes usually recede to a more lateral position than in the P.S. animals.

The pad at the base of the bladder in the N.P.S. female diminishes in size during the early days of life. In most cases it

disappears by the ninth post-natal day, and is never found after the thirteenth day. The following description will, therefore, apply only to the further development of the prostate gland in P.S. females.

With the disappearance of the connecting bridge and with advancing age, the prostatic lobes, which in the early days of life had been almost round, now begin to elongate in a cranio-caudal direction and become oval in shape. Continued growth

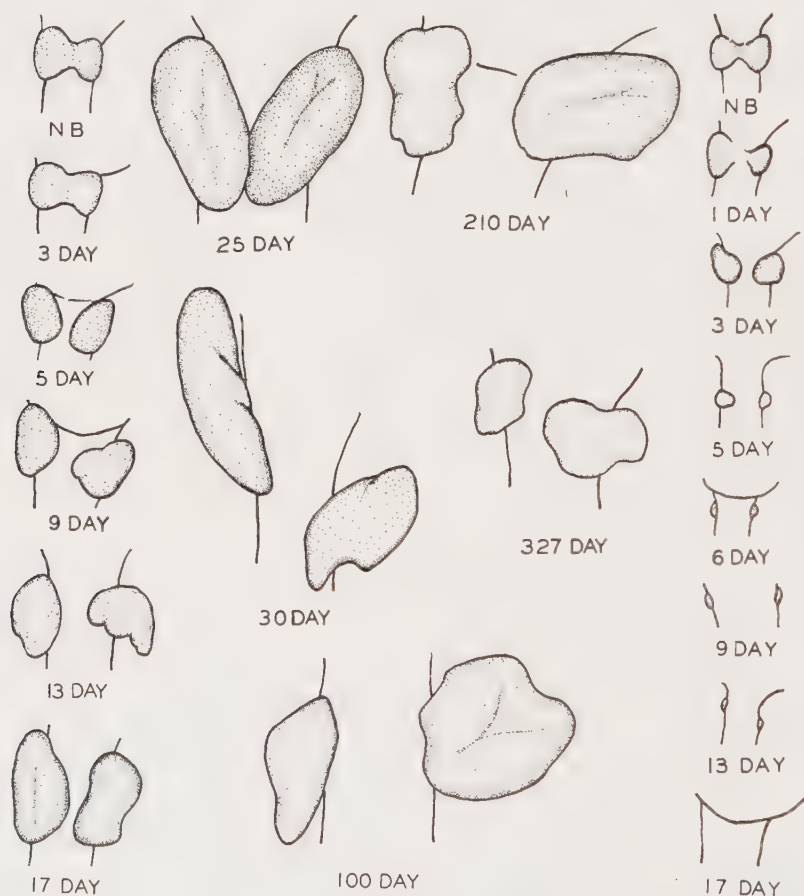


Fig. 2 Camera lucida drawings of post-natal stages in development of female prostate, and regression of stromal pad in females of non-prostate strain. Right hand column shows disappearance of pad in non-prostate strain. $\times 10$.

causes not only an increase in size, but a more irregular gland shape, and most specimens over 9 days of age show some lobulation of the outer gland margin.

Changes in the macroscopic appearance of the prostate are relatively slight after the age of 15 days. There is considerable variation among individuals as to size of the gland, but in general, the maximum size is reached around the thirtieth day of age. In older animals the lobes tend to shift dorsally and come to lie with their greater portion along the lateral vaginal wall.

After the hundredth day of life the lobes often become thinner and somewhat more transparent. In older animals they may be reduced in size, but here again the variation among individuals is large, and occasionally an old female will be found with a prostate as large as that of a 30 day female.

Microscopic

While it is impossible to distinguish macroscopically between the thickened pad found at the base of the bladder in the newborn N.P.S. female and the true prostate of the P.S. female, microscopic examination reveals an important difference (figs. 5 and 6). Only in the prostate strain do the two embryonic primordia of the gland unite. The distal ends of one or two of the urethral epithelial buds grow into the mesenchymal pads where they branch and soon begin to form glandular tubules. No glandular tissue is ever seen in the thinner connecting bridge between the lateral condensations.

Microscopic examination of the newborn N.P.S. female shows that although the stromal pad is present at the base of the bladder, the epithelial cords, although they may be present, never enter it.

Despite the fact that the number of buds tended to increase during embryonic life, the newborn P.S. female usually has but two, or in some cases three, buds on each side of the urethra.

The stromal pad, which lies at the site of the future prostate gland is some distance cranial to the site of origin of the epithelial buds. For convenience, the buds will be referred to in

the order in which they grow out from the urethra, beginning at the most cranial one. In most cases bud II alone enters this mesenchymal area. In a few cases bud III may enter instead of bud II, and less frequently both cords reach into this area (table 2). The remaining buds stay within the urethral sheath,

TABLE 2
Position of definitive prostatic duct among urethral cords in post-natal females of the prostate strain

AGE (DAYS, P.N.)	NO. OF RIGHT PROSTATE BUDS	DEFINITIVE DUCT	OTHER BUDS	NO. OF LEFT PROSTATE BUDS	DEFINITIVE DUCT	OTHER BUDS
NB ¹	2	II	CRAN ²	2	II	CRAN
NB	3	II	1) CRAN 1) CAUD ³	2	II	CRAN
NB	3	I & II	CRAN	3	II	1) CRAN 1) CAUD
NB	2	II	CRAN	2	None	
1	3	I & II	CAUD	3	I	2) CAUD
1	3	III	2) CRAN	1	I	
3	2	II	CRAN	2	II	CRAN
3	1	I		1	None	
4	2	II	CRAN	2	II	CRAN
5	1	I		1	I	
5	4	II	1) CRAN 2) CAUD	3	II	1) CRAN 1) CAUD
5	3	III	2) CRAN	3	III	2) CRAN
9	2	II	CRAN	3	III	2) CRAN
10	1	I		2	I & II	
13	2	I & II		2	II	CRAN
14	2	I & II		2	I	CAUD

¹ NB = newborn.

² CRAN = cranial.

³ CAUD = caudal.

ending blindly in the mesenchyme surrounding the urethra (see fig. 6, Witschi, Mahoney and Riley, '38).

The histological picture of the epithelial cords of the newborn female is quite similar to that of the late embryo.

Many of the 1-day female prostates are characterized by definite signs of coiling and branching of the cord or cords that enter the lateral stromal pads. The branching occurs in only those parts of the cords that enter the stromal pad. Prior to

entering this area the cord runs cranially in a relatively straight line through the mesenchyme within the urethral sheath.

During the following 10 days the prostate gland becomes more complex. The cord or cords entering the stromal condensation become extremely branched, with secondary fusions of branches, and many short outgrowths (fig. 3). With the increase in the amount of glandular tissue in the prostate, the mesenchyme becomes relatively more scanty.

The cord that enters the definitive gland site becomes enclosed at its lower end by several layers of fibrous connective tissue. This cord becomes emphasized at the expense of the

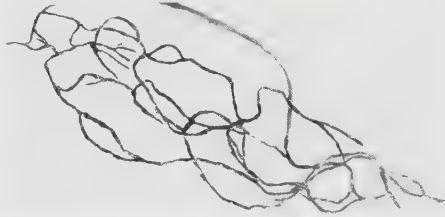


Fig. 3 Clay reconstruction of branching undergone by epithelial cords in left prostatic lobe of a 5-day female of the prostate strain. Ventral view. Cranial end to reader's right, caudal to reader's left. Outer (distal) edge of lobe at lower side of picture, inner (medial) edge of lobe and definitive duct at upper side of picture. $\times 15$.

remaining buds, which gradually become shortened. In most animals the definitive duct begins to acquire a lumen at 5 days of age.

The cords in the N.P.S. females do not branch or coil and they never acquire lumina. In animals of this strain over 10 days of age, no true prostate buds are observed.

At 10 days of age in P.S. females the duct has acquired its lumen. The tubules of the glands are likewise beginning to show lumen formation, a process which progresses from the duct distally (fig. 7). Concomitantly the glandular cells take on a columnar shape with round or slightly oval basal nuclei, and commonly one nucleolus. The tubule walls are made up

of single rows of these tall cells. The wall of the duct is made up of cuboidal epithelial cells, and surrounded by an external connective tissue sheath. The cords which do not reach the gland site in many cases also show lumina. The intertubular stroma becomes more sparse, and empty areas begin to appear between tubules in the gland.

Between 15 and 20 days of age the tubules continue to acquire lumina which in many cases become quite wide. The intertubular tissue begins to show strands of smooth muscle. In many animals the definitive efferent duct is the only one of the several embryonic epithelial buds to persist. If rudimentary, blind ducts persist, they open into the urethra either caudal or cranial to the main duct (see table 2). In a few instances rudiments remain both above and below the definitive duct.

By the twenty-fifth day of life most of the tubule cells show a light area lying between the nucleus and the lumen end of the cells (figs. 8 and 10). This light area is similar to that found in the epithelial cells of the normal male prostate.

Between the twenty-fifth and thirtieth day the tubules still increase in size, continue to branch, and come to fill almost the entire gland. As a result the amount of intertubular tissue becomes very reduced. Each tubule is now surrounded by two or three layers of fibrous connective tissue and many are distended with secretion. The tubular epithelium now appears more cuboidal than columnar (figs. 9 and 11) but light areas are still present in the cells.

Between the thirtieth and fortieth days of life the prostate gland of the female begins to undergo involution. The epithelium in all but the smallest tubules becomes flattened, and light areas disappear, even in the cytoplasm of the cuboidal cells. The amount of intertubular tissue increases, and most tubules lose their smooth, rounded outline. This description applies equally well to all later stages examined. The gland persists for the whole lifetime of the animal but continues to present this involuted appearance.

DISCUSSION

The development of the prostate in the female rat shows two distinct stages. The first stage, including the entire embryonic period and about the first 30 days of post-partum life consists of normal development of a glandular structure histologically similar to the ventral lobes of the prostate of the male. Epithelial buds grow out, enter a stromal pad, branch, form anastomoses, and change from solid cords into tubules with secretory epithelia.

At about 30 days of development, however, involutional changes set in. These changes, as suggested by Price ('39) closely resemble the histological picture of the involution taking place in the male prostate subsequent to castration. Moore, Price and Gallagher ('30) consider the light areas in the epithelial cells as denoting secretory activity in the male prostate. In adult males they disappear after castration. Korenchevsky ('37) and Witschi et al. ('38) have noted the similarity of the female prostate to that of the castrate male, and this investigation shows the development of the castrate condition during the fourth and fifth weeks post-partum. It has also been shown by Korenchevsky and by Witschi and co-workers that the prostate of the female rat reacts to sex hormones essentially as does that of the male castrate.

Price ('36) reports that castration of male rats during the first week of post-natal life does not prevent the development of a prostate, which at 30 days, shows distended lumina, high epithelium, and characteristic light areas in the epithelial cells. Involution does not set in until after 30 days of age, after which time the castrate picture begins to appear.² She gives three possible explanations for this phenomenon, "1) removal of the young testes may not result in such rapid disappearance of the testis hormone as in the adult; 2) some unknown factor perhaps genetic or hypophyseal may act upon the glands up to puberty at which time they may become exclusively de-

² Unpublished work of Witschi indicates that castration of newborn male rats results in somewhat smaller prostates at 30 days of age. The general picture of the gland, however, is as described by Price.

pendent upon male hormone alone; or 3) the glands may be determined by male hormone before the time of castration.” In 1939 the same author suggested that the adrenal glands may maintain the prostate of the young male castrate and of the young normal female, prior to the time at which involution has been observed to set in.

As Price has shown, the picture of prostate development in the castrate male and in the normal female is so similar that one is tempted to infer some similar-acting mechanism. Pfeiffer ('36) has shown that the ovary does not produce male hormone, or if it does, only sub-threshold amounts are formed. Assuming the developmental mechanisms of male and female prostates prior to 30 days of age to be similar, the first and third of the original assumptions of Price seem thus to be ruled out. It therefore appears that the prostate is independent of the gonad hormone prior to puberty. Up to this stage development in the two sexes is similar, but beyond this critical period, male hormone apparently is necessary to maintain the normal state of the gland. Therefore, involutional changes begin in the castrate male and in the normal female during the second month.

Price ('36) in describing the embryology of the prostate homologue in the female, states that the prostatic cords coil, but do not branch, prior to 5 days post-natal development. The discrepancy between this report and the very distinct branching of the cords noted in some animals under that age in this investigation may be due to the fact that Price obviously was not working on a strain of rats with a very high incidence of prostates in the females. The enormous difference which we found between the 5-day P.S. female and the 5-day N.P.S. female illustrates the point. Her material apparently stood somewhere between these two extremes, and thus some difference in degree of development at certain stages may be expected.

Witschi, Mahoney and Riley ('38) on the basis of study of the female gland in the adult condition, suggest the homology of the female prostate to that part of the male ventral prostate

which arises from the most ventral of the five efferent ducts. The fact shown here that the female prostate in most cases arises from a single bud seems to justify the assumption that the gland in the female usually is the homologue to one-fifth of the male ventral lobe. However, in certain females two ducts drain a lobe and here the gland would appear to be homologous to two-fifths of the ventral lobe of the male prostate. Variations among individuals as to which bud becomes the definitive duct hinder attempts to homologize the ducts in the male and female glands.

Raynaud ('38) postulates the production of male hormone by the female organism in order to explain the continued presence of prostatic lobes after birth in intersexual female mice obtained by injections of male hormone into the mother during pregnancy. Hamilton and Wolfe ('37) also conclude that the presence of prostatic lobes in the normal female indicates the occurrence of male hormone substances in the female. However, in the rat at least, if this alone were the case, it is difficult to account for the relatively low occurrence of the gland in females injected with male hormone during embryonic or post-natal life, as listed by various authors (Hamilton and Wolfe, '37; Korenchevsky, '37; Greene, Burrill and Ivy, '38).

The situation in the mouse seems to be different in this respect. Both Raynaud ('38) and Turner ('39) report almost 100% occurrence of the female prostate in animals born of male sex hormone-treated mothers.

In sharp contrast to the comparatively infrequent occurrence of female prostate glands among male hormone-injected female rats, is the 93.0% appearance of the gland in the normal females of the prostate strain here used. Since this strain of rats was developed from a colony showing a female prostate incidence of only 26.7% merely by selective breeding, it appears that genetical factors are more effective than hormonal ones in causing the appearance of the gland.

This investigation demonstrates that there are two types of females with respect to the occurrence of prostate glands after 25 days of age. The first class shows a steady development

from the first appearance of the gland up to about the thirtieth day of post-natal life. From this time on it gradually undergoes involution, but remains visible in most cases. A second group is made up of females in which a true prostate is never formed. In some cases only the stromal pad appears, and no epithelial buds ever arise from the urethra to form the prostatic tubules. In other cases, some buds make their appearance but they never unite with the pads, and in all cases, neither one of these primordia is seen after the second week of post-natal life.

SUMMARY

1. The prostate gland of the female rat arises from a double primordium which first appears at 19 days 8 hours of embryonic life. At this stage it is characterized by solid epithelial cords arising laterally from the urethral epithelium, and also by a stromal condensation lying ventral to the urethra. In the last pre-natal stage these cords extend to the inner edge of the urethral sheath, and for some distance cranially in the mesenchyme along the urethra, inside the sheath.

2. In the newborn female, one, or at the most, two, of the cords are growing out into the stromal pad. Further post-natal growth consists of coiling and branching of the definitive duct forming the glandular area, reduction of the remaining prostatic buds to short outgrowths from the urethral epithelium, and gradual formation of a lumen in the duct and the glandular tubules arising from it.

3. The gland of the 30-day female has reached its maximum development morphologically, although there may be some further increase in size. The tubules all have lumina, the larger ones distended with secretion and with epithelium ranging from tall columnar to low cuboidal, depending upon the amount of distention of the tubules. Light areas appear in the epithelial cells, distal to the nucleus which lies basally.

4. After this stage of maximum development the female prostate gradually undergoes involution, and comes to resemble the prostate of the castrate male. The amount of inter-

tubular tissue increases, the epithelium becomes lower and loses its light areas, and the tubules become more irregular in cross sections. This condition continues throughout the remainder of the life of the animal.

5. In the female rat, two classes can be distinguished on the basis of the occurrence of a prostate gland. One group (P.S.) shows both embryonic rudiments, and these develop into the true prostate gland. The second group (N.P.S.) may or may not, show both embryonic primordia of the gland, but if both are present they never unite to form a true prostate. Whether only the stromal pad ventral to the urethra develops, or both the pad and the embryonic urethral buds appear, by the third week of life no rudiment remains.

6. The prostate of the female rat is shown to be homologous to a part of the ventral lobe of the prostate of the male rat, in most cases being comparable to one-fifth of the male lobe. Due to variation in the bud number and position in the two sexes, the homology of the urethral buds in the male and the female is not clear cut.

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PLATE 1

EXPLANATION OF FIGURES

- 4 Cross section through urethra of 19 day 8 hour female embryo of prostate strain. Left epithelial bud arising from dorso-lateral urethral wall. $\times 250$.
- 5 Cross section through prostate of 5-day female of prostate strain. Distal ends of epithelial cords in bilateral stromal pad, prostatic ducts in urethral sheath. $\times 55$.
- 6 Cross section through stromal pad of 5-day female of non-prostate strain. Stromal pad devoid of epithelial cords. Compare with figure 5. $\times 55$.
- 7 Cross section through prostate area of 15-day female. Note lumen formation beginning in epithelial cords. $\times 55$.
- 8 Cross section through prostate of 25-day female. Note large lumina, secretion in tubules. See also figure 10. $\times 55$.
- 9 Cross section through prostate of 35-day female. See also figure 11, and compare with figures 8 and 10. $\times 55$.

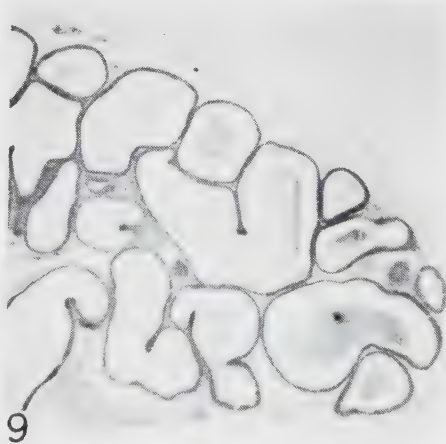
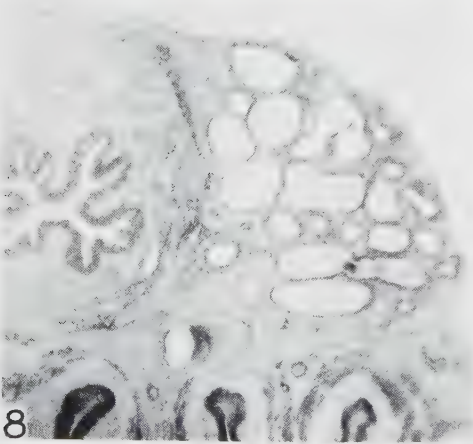
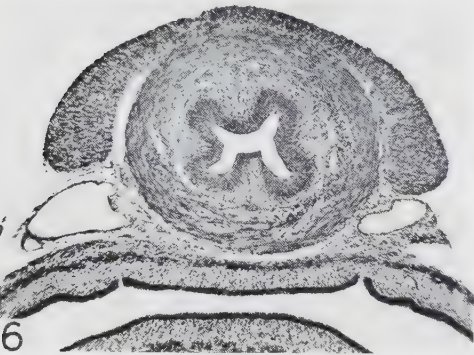
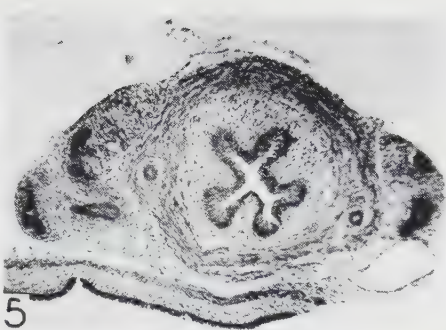
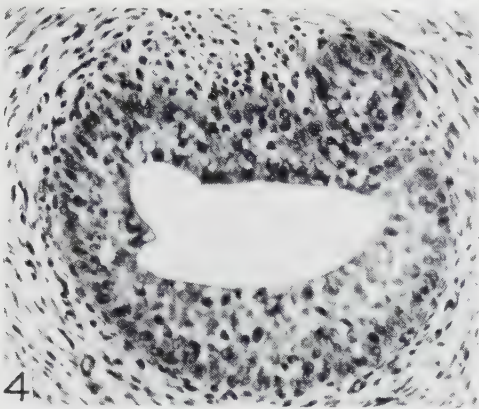
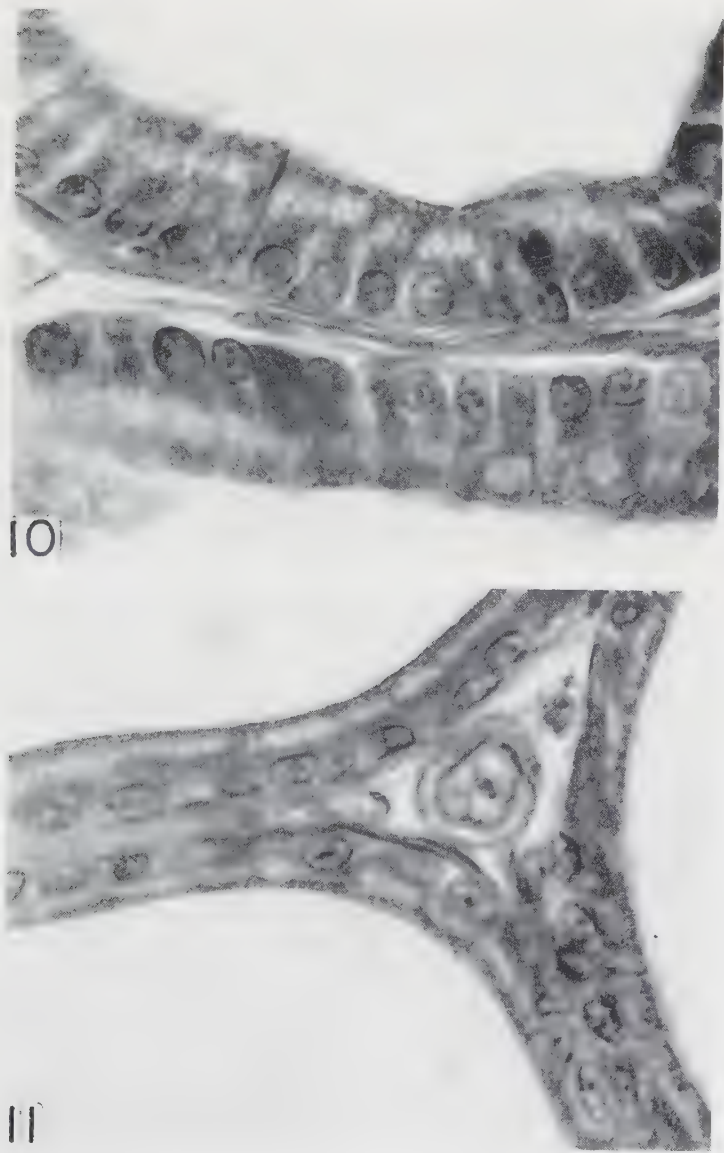


PLATE 2

EXPLANATION OF FIGURES

10 Cross section of tubule wall of prostate of 25-day female. Note columnar epithelium, basal nuclei, light areas between nuclei and tubule lumen. $\times 800$.

11 Cross section of tubule wall of prostate of 35-day female. Note cuboidal epithelium, light areas still remaining in cells. $\times 800$.



SOME OBSERVATIONS ON THE PRE-ADIPOSE CELLS

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SEVEN FIGURES

In regard to the origin of the fat cells it is generally believed that they arise from connective tissue. Opinions differ as to the type of connective tissue from which the fat tissue arises. One group of workers represented by Flemming believes that fat cells arise from general connective tissue while the other group represented by Toldt, claims that the origin of the fat cells is a special connective tissue developed from special organs and that all further formation of adipose tissue is derived from the outgrowth of these organs. Waldeyer holds a compromising view stating that fat cells are developed both from special and ordinary connective tissue cells. Hammar distinguishes two types of fat tissue formation; the primary type forms a well defined lobule (primary organ), the secondary type forms fat without primary organs. Shaw considers two possibilities with regard to the origin of the fat cells: (1) that they are cells of the connective tissue differentiated to form and store fat, (2) that they are ordinary connective tissue cells temporarily laden with fat. Bell states that pre-adipose tissue may or may not form a special lobule. This pre-adipose tissue, according to him, belongs to the ordinary fibrillar connective tissue. In this respect he supports Flemming's view.

As to the manner of the transformation of connective tissue cells into fat cells, Flemming observes that connective tissue cells lose their connections with each other and assume a somewhat round shape and that the amount of their protoplasm increases. The nucleus comes to lie on the side of the

cell either before the fat granules are developed or just after they are beginning to appear. In either case the fat soon collects as one main globule with small ones around it. Hammar states that the cells may develop a large amount of protoplasm before the deposit of fat begins or they may accumulate fat without any marked increase of protoplasm. Bell found that in the cat, the primitive organs consist of a well defined mass of closely packed cells rich in protoplasm, but that in the calf, dog and man these structures are composed of a reticular tissue with branched cells. The branched cells of the pre-adipose tissue may contain fat droplets long before any true adipose is present. Löwe describes the presence of granules prior to the deposition of fat. Polijakoff found in white rats a large number of granular cells in the region where fat is being deposited. These granules, according to the same author, are made of fat combined with some albumoid. Metzner claims the actual transformation of protein granules into fat. Jordan believes there are two types of fat cells present in the body. One type is found in the subcutaneous tissue, the cells of which elaborate one or several gradually enlarging fat globules; the other type is present along the blood vessels, and consists of ovoid mesenchymal cells with dense granular cytoplasm. The granules liquefy, giving the cytoplasm an alveolar appearance.

From the above statement, the characteristics of the cells prior to becoming fat cells can be summed up as round, ovoid or branched in shape, rich in protoplasm which may be granular, having a rather deeply stained eccentric nucleus and tending to develop in groups especially along the blood vessels. Are these cells really a special type of cell that can be called lipoblasts or just ordinary cells of another type? The above mentioned characteristics are quite homologous with those of the macrophages. The cells described by various authors may be nothing more than macrophages which are still multipotential in nature and capable of differentiation into various types of cells. In order to prove this possibility, the following experiments were performed. In the first experiment, rabbits

were starved for a period of 1 week to deplete the fat in the hope that some new fat cells might be formed after refeeding. Macrophages were ear-marked by injection into the abdominal cavity of 1 cc. 2% lithium carmine in the physiological saline solution every other day for 2 weeks at the time of refeeding. Then the animal was killed and thin portions of the omentum were cut out and made to spread. They were fixed in Zenker-formol and stained with Harris hematoxylin. In the specimens

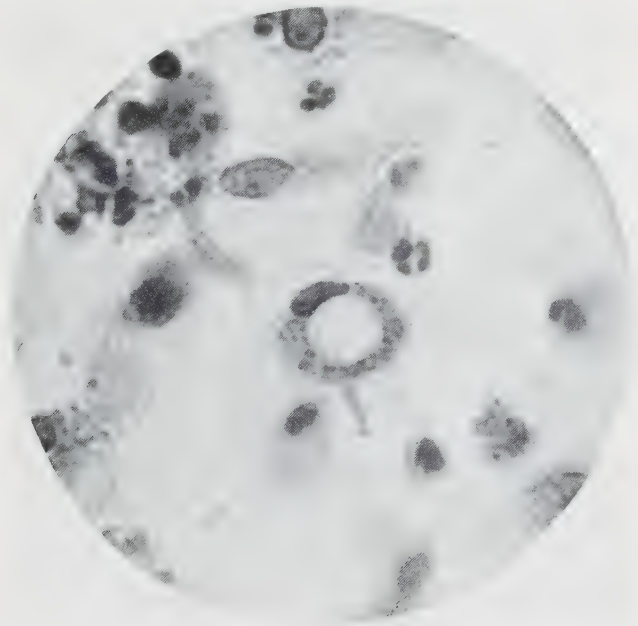


Fig. 1 Photomicrograph, showing a young fat cell from the omentum of the rabbit with dye granules in its cytoplasm.

thus obtained most of the macrophages were seen to be vitally stained by lithium carmine, the dye being also found in the cytoplasm of some of the fat cells (fig. 1). In some specimens, due to irritation caused by the dye, some of the macrophages hypertrophied to form giant cells containing several nuclei. This polynuclear condition was also found in many of the fat cells (fig. 2). The nuclei of the polynuclear fat cells in some

cases formed a heap on one side of the cell and in others, were evenly distributed around the fat globule (fig. 3). The transitional form between the fat cells and the polynuclear giant cells has also been found as a giant cell containing fat vacuoles of various sizes (fig. 4). Coincidentally neutrophil leucocytes were also found in large number and the fragments of the nucleus of these cells or even the whole leucocyte were also

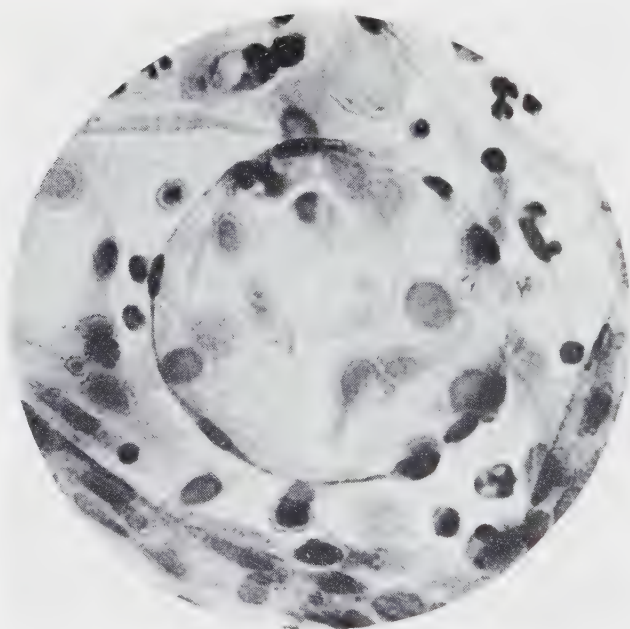


Fig. 2 Photomicrograph, showing a polynuclear fat cell from the same specimen with several flattened nuclei around the cell.

seen in the cytoplasm of the hypertrophied macrophages as well as in that of the polynuclear fat cells (fig. 5). In one cell as many as ten leucocytes were counted in the cytoplasmic mass (fig. 6).

The second experiment was made on the rabbit's bone marrow. The animal was injected subcutaneously with equal parts of benzol and olive oil at the dosage of 1 cc. of pure benzol per kilogram of body weight of the rabbit. The injection was done

every day for 1 week in order to produce depletion of the bone marrow. When the leucocyte count of the animal rose, showing a sign of regeneration of the bone marrow, the animal was killed and pieces of marrow were spread on the slide and stained with Harris hematoxylin. In some of these specimens the newly formed fat cells as well as the macrophages are seen

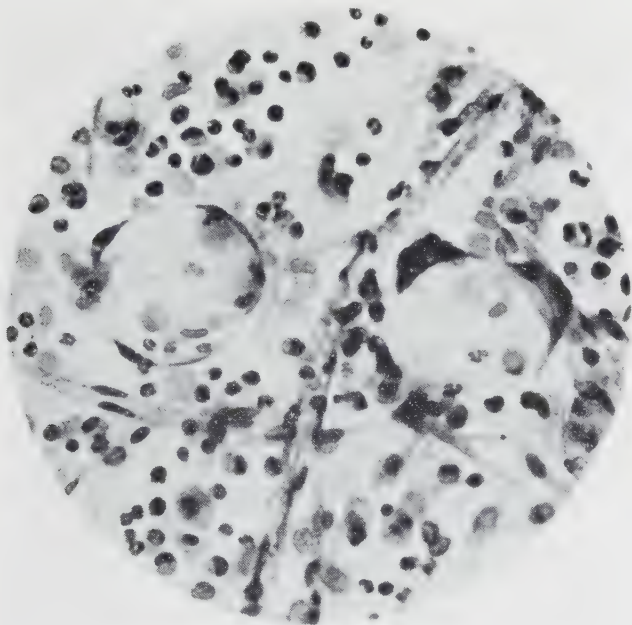


Fig. 3 Photomicrograph of two polynuclear fat cells, in one of which the nuclei are distributed evenly around the fat globule and in the other the nuclei form two masses on the side of the cell.

to contain hemosiderin formed by destruction of the erythrocytes caused by the drug (fig. 7). The appearance of hemosiderin in the cytoplasm of the fat cells is interpreted to mean that the fat cells have just come through the stage of being macrophages, at which time this substance was phagocytosed. It is, therefore, obvious that the cells designated by various authors as pre-adipose cells are by no means a special type but just ordinary macrophages which are also a derivative of

the primitive connective tissue cells. If this observation proves to be correct, the theory claiming that fat tissue arises from a special connective tissue must be disclaimed, as macrophages can be found in the tissues all over the body. They may be either scattered or in groups. Since the macrophages are said to arise both from fixed connective tissue and blood cells (monocytes) the origin of the fat cells cannot be strictly

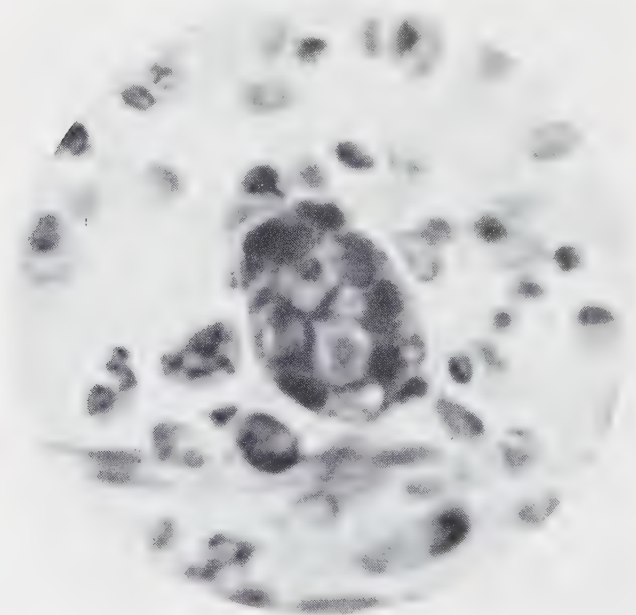


Fig. 4 Photomicrograph, showing a giant macrophage with several nuclei and fat globules—a transitional form between macrophages and fat cell.

limited to connective tissue. Many facts reported by various authors would support this hypothesis.

Neal and Ellis, in studying fat necrosis of the omental tissue following the intraperitoneal injection of lipase, have found that the area of fat necrosis is always surrounded by a margin of undamaged fat cells. According to the same authors, several hours after the injection, there was a zone of leucocyte demarcation. Thirty hours after injection, endothelial leuco-

cytes and giant cells made their appearance and later increased in number. Five or 6 days later, neutrophil leucocytes disappeared but giant cells and endothelial leucocytes remained in the place. From the above statement, the question will arise whether the zone of undamaged fat is the remains of the most peripheral portion of the original fat tissue or fat newly formed around the damaged fat tissue. In view of the

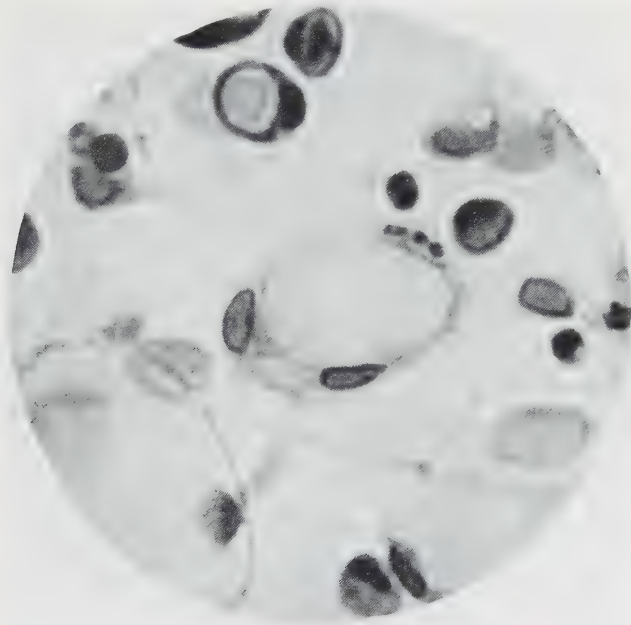


Fig. 5 Photomicrograph of a young fat cell containing a polymorphonuclear leucocyte at one side of its cytoplasm.

fact that the lipase is not injected intravenously but intraperitoneally it is highly possible that this zone of fat is formed after the destruction of the original fat tissue as the lipase acts from the periphery of the fat tissue instead of from within and along the course of the blood vessel. If this fat is newly formed, its probable source of origin is the endothelial leucocytes, in other words, the macrophages which surround the area after the disappearance of the neutrophil leucocytes. The

concentration of the macrophages around the damaged area is to remove the debris, but some of them by taking in the fatty substance produced by lipase, become new fat cells.

Ganfni in studying the formation of the appendices epiploicae of the large intestine has found that connective tissue cells surrounding the blood vessels became transformed into two types of cells, one type resembling the reticular cell the

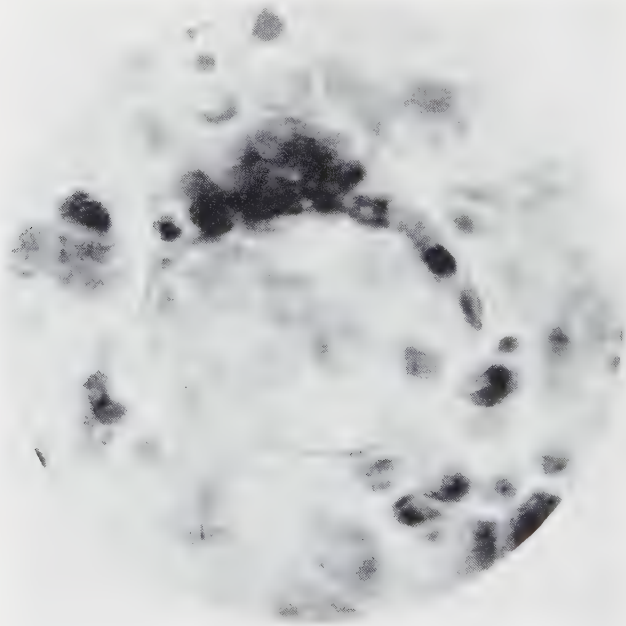


Fig. 6 Photomicrograph, showing a polynuclear fat cell from the omentum with several ingested polymorphonuclear leucocytes (right upper part).

other, lymphatic cells. The lymphatic cells later developed into fat cells. They are gradually filled with fat globules which are first very minute and finally become large. The lymphatic cells, so-called by this author, are probably young macrophages which resemble, in many respects, the lymphocytes. Klein also found that adipose tissue is preceded by an accumulation of the lymphocytes forming masses of lymphoid tissue which accompany the blood vessels.

Rutledge and Latta in their experiment with the intraperitoneal injection of trypan blue in the rat found that some of the fat cells had lost their fat content and were transformed into cells resembling macrophages which took dye like other macrophages. They did not suggest macrophages as the origin of fat cells but, the reverse—the possibility of the transformation of fat cells into macrophages. It is not improbable that

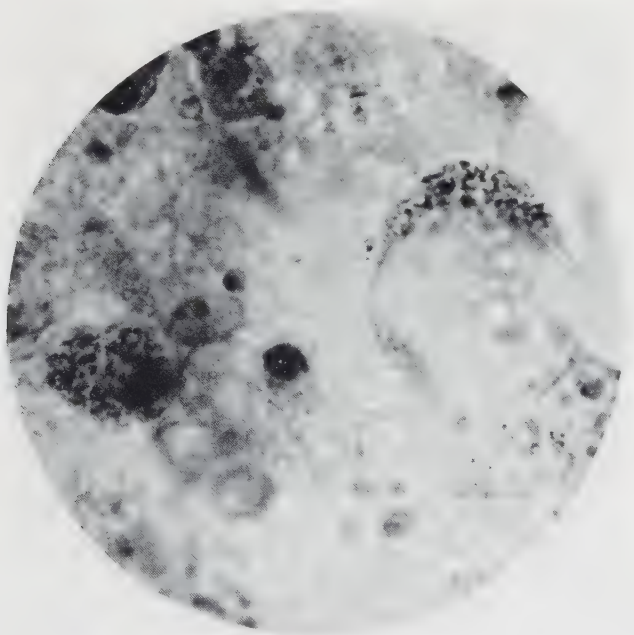


Fig. 7 Photomicrograph, showing a fat cell from the bone marrow of rabbit after being treated with benzol containing hemosiderin granules in its cytoplasm. A similar macrophage is shown at its left side.

fat cells return to their original form of macrophages by losing the fat content. Whether or not a mature fat cell is still able to store dye in its body is unknown. What Latta and Rutledge saw in their specimen might have belonged to a fat cell which had already returned to the condition of a macrophage or recently formed from a macrophage which had already stored dye in its body.

A mature fat cell, on account of the stretching of the cytoplasm, may have changed in viscosity, becoming impenetrable for the dye. In our specimen where many mature fat cells were crowded, neither storage of dye nor ingestion of leucocytes could be found in the cytoplasm of the fat cells. The dye was only found in the macrophages lying among the fat cells.

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THE CONTENT AND DISTRIBUTION OF MINERALS IN HUMAN AMNION AND CHORION AT TERM

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ONE PLATE (SEVEN FIGURES)

The importance of the metabolism of certain minerals, particularly calcium and iron, during pregnancy has long been recognized and extensively studied. To the extent also that histological methods have permitted, attempts have been made to localize certain minerals within the organs of the developing fetus and the extra-embryonic membranes. It has been possible to do this, but not with a high degree of precision, since the methods used depended for their success on intensity of staining reaction or on microchemical reactions that presented diffuse pictures microscopically. Nevertheless, by such means it was found, among other facts, that iron may be precipitated as granules at the boundary between villus epithelium and connective tissue, and calcium may accumulate in syncytial cells of the villi and to some extent in the amnion and chorionic plate.

To permit a closer estimation of the mineral structure of the cell than is possible by any staining technique, the micro-incineration method was used. In brief, the technique consists of fixing tissues in a mixture of absolute alcohol and formalin, cutting paraffin sections at 4 μ or less, and ashing the sections by exposing to gradually rising temperatures up to 650°C.

¹ The work here reported was aided by a grant from the Carnegie Corporation of New York.

Left on the slide then is a non-volatile, mineral ash which is resistant to the highest temperature obtained. In general, the outline pattern of the original tissue can be recognized. A cover glass is placed over the preparation and sealed without a mounting medium, and observations may be made by means of darkfield illumination. The method, together with an interpretation of its results, has been given in detail by Scott ('33), and we have followed in this work the technique there recommended.

Iron and iron-containing minerals present as Fe_2O_3 can be determined with fair accuracy by color alone since they will appear as particles of varying shades of yellow and red (Policard, '23). The apparent presence of iron was verified in most of the following observations by either the prussian blue or the tannic acid test. Heat-resistant calcium may be present in several forms—among them CaO , $\text{Ca}_3(\text{PO}_4)_2$ and in combination with silica. All these calcium-containing ash residues are white or whitish, monorefractive and usually very brilliant. For purposes of verification, material interpreted as calcium in the following observations was subjected to one or several microchemical tests, each of which was sensitive and gave clear-cut results. The two following were chiefly used—treating the calcium-containing ash with N/10 HCl and N/10 H_2SO_4 to obtain crystals of calcium sulfate, and the alizarin test in which a red precipitate is obtained with sodium alizarin sulfonate. By means of this latter test it was possible in many instances to localize accurately within the cells the calcium-containing ash particles. No attempt was made at this time to identify with certainty other minerals. The material used was from eight mature placentas obtained in the delivery room from clinically normal cases delivered without complications.

At term the epithelium of the amnion is cuboidal everywhere except in the placental portion over the chorionic plate where it tends to be columnar. Very early in fetal life columnar cells may be found in all parts of the amnion though this is by no means the rule. There is, however, a diminution in

the height of these cells with advancing gestation and this decrease begins at about the time of fusion of amnion and chorion—usually about the second month of intrauterine life. The chorionic epithelium, on the other hand, rapidly becomes low cuboidal (Langerhans') or squamous and remains so until the end of pregnancy.

On incinerating a section from the free, or non-placental, portion of the amnion (fig. 1) the cuboidal epithelial cells are seen to be rich in both coarse and fine ash particles that are usually irregularly distributed in the cells. Around the nuclear membrane, however, there is always a heavier condensation of ash of a particularly luminous kind. Within the nucleus there is a small amount of ash, most of it white though occasionally there are small pale yellow granules indicating iron. Very striking is the presence of ash in rather regular formation at the base of the cells. This line of granules can be shown to lie within the cells and does not represent the basement membrane. The latter can be seen in adjacent sections stained with hematoxylin and eosin, but in the incinerated sections the basement membrane leaves absolutely no mineral residue and must be regarded, therefore, as a formation free of inorganic mineral salts. A dozen or more cells in a row are frequently seen in which there is no indication of ferrous ash, the granules within the cytoplasm being dull white. Whenever iron does occur, it is present as one, or very infrequently as a small cluster of minute, yellow or pink-colored particles. There is striking lack of uniformity in the quantity of heat-resistant material present in the cells. Rarely does one see within a single field cells whose ash content is the same. The connective tissue below the epithelium is loose and contains varying amounts of heavy collagenous fiber bundles. These bundles are represented by a dense white ash, while the occasional elastic fibers are invariably poor in ash.

In its placental portion (fig. 2) the amnion shows characteristic columnar cells whose nuclei frequently lie at or near the distal pole of the cell. In these cells there is an especially large accumulation of white ash around the nuclear mem-

branes, and the nuclei themselves are somewhat richer in ash than the nuclei of the cuboidal cells of the free portion of the amnion. Very striking is the deposition of ash at the boundaries between adjacent epithelial cells. The bases of the cells, on the contrary, are usually totally devoid of mineral residue of any kind. Cytoplasmic vacuoles are frequently found whose contents are also devoid of mineral residue. It is observed also that there is no ash left by the basement membrane. The connective tissue beneath the placental amnion is distinctive in that it contains fewer cells than the connective tissue of the free amnion, as well as far fewer elastic fibers.

To what extent the calcium ash is water-soluble was determined on a series of sections of both amnion and chorion. Usually very little calcium ash of the amnion goes into solution in water, and none of the iron-containing ash is lost in this way. However, occasional sections are encountered in which approximately half of the perinuclear residue is found to be soluble while most of the intracellular ash remains intact (fig. 3). The squamous epithelium of the chorionic surface is affected by treatment with water far less than the amnion, but the intervening connective tissue may lose relatively large amounts of its calcium ash, with the result that large clear areas are produced. It should be noted, however, that there is some variation in the amount of water-soluble ash in the connective tissue.

Most of the mineral residue that is not water-soluble is acid-soluble. On treating with N/10 HCl it is possible to remove from the cells of the amnion almost all of the perinuclear calcium ash as well as some of the more scattered mineral ash. But almost invariably there remains a more resistant material, arranged usually along the lateral walls of the cells (fig. 5), and sometimes along their basal and free margins (fig. 4). Most of the connective tissue ash is also lost except that which is associated with the elastic fibers and is not so easily dissolved. The amniotic membrane from the surface of the placenta usually loses far more ash on treating with either water or acids than the free amnion. The maxi-

imum amount of ash can be removed by treating first with water and then with HCl. After such treatment the ash loss may be so great that the nuclei can only be discerned with difficulty. On treating with both HCl and H_2SO_4 the ash is removed to such a degree that identification of the tissue becomes impossible. There are left, however, scattered granules of very dull white and bluish ash whose identity could not be determined with certainty but which suggest silica.

The mineral content of the chorionic epithelium is essentially equal and uniform in all parts. Figure 6 may be taken as characteristic. It shows a portion of the epithelium together with a small amount of connective tissue and adherent decidual cells. The epithelial cells show prominently, both fine and coarse, brilliant white granules at the cell membranes with a small accumulation of ash with similar properties at the nuclear membranes. The large decidual cells have their nuclei well outlined, and the normally homogeneous or finely granular cytoplasm, as seen in hematoxylin and eosin stained sections, is duplicated in the incinerated cells by a peculiarly homogeneous-appearing mineral residue. Most of these cells are deep blue in color when seen under darkfield illumination. Cytoplasmic vacuoles, when present, are wholly free of ash. The connective tissue cells are large and densely filled with ash and contain a small amount of iron. The chorionic epithelium and the decidual cells are also very poor in this mineral. On treating such a section with water, almost no ash is lost from either chorion or decidua, but relatively more is dissolved out of the connective tissue layer. On treating with N/10 HCl, however, only the peripheral ash of the chorionic cell layer remains and the same is true of the connective tissue cells. The calcium-containing residue of the decidua is slightly more soluble in acid than that of the chorionic epithelium, and its soluble ash is also far more evenly distributed through the cells.

The above observations conform in general with the findings of others on various types of epithelium. Epithelia are usually rich in minerals. This was shown for placental epithelium

by Schoenholz ('29), for ependyma by Alexander and Myerson ('37), and by many others. There is, however, much variation in the location of the minerals, for Alexander and Myerson report most of the ash as being present in the ependymal cell nuclei, whereas in the amnion the present preparations demonstrate that the nuclei contain little or no ash. However, there should be no reason to anticipate uniformity in this regard, because the physiological activities of different types of epithelial cells are quite variable. At term the cytological picture of the amnion is usually one of secretory activity which is apparent also in the incinerated sections (fig. 2). While fat, glycogen, iron and calcium have been described in amnion and chorion, there is divergence of opinion regarding the relative importance of secretion and storage in accounting for the presence of the first two and less regarding the latter two. In any case, it seems probable from the incinerated specimens here described that at term the peak of amniotic secretory activity has passed. Polano ('22), availing himself of staining methods, described at the end of pregnancy iron and other constituents of the amnion and vacuoles of various sizes that sometimes fill the entire cell in the placental part of the amnion. In view of the total lack of mineral ash in such vacuoles in the present specimens of late gestation, it is not likely that they are at all concerned with mineral metabolism.

SUMMARY

1. The cytoplasm of the non-placental part of the amnion is rich in minerals, the nuclei poor. The quantity of water-soluble minerals, including calcium, in the cytoplasm is low, although this varies in different specimens. There is very little iron in the cytoplasm, less in the nuclei.

2. Most of the minerals in the placental portion of the amnion are present just within the cell membrane, except at the base of the cell which is invariably ash free. Minerals are absent from all cytoplasmic vacuoles.

3. The basement membrane of the amnion is a mineral-free structure.

4. A small amount of a mineral—probably silica—is present in the epithelia of both amnion and chorion.

5. The decidual and connective tissue cells are rich in water-soluble minerals, and contain more iron than either the amniotic or chorionic epithelium.

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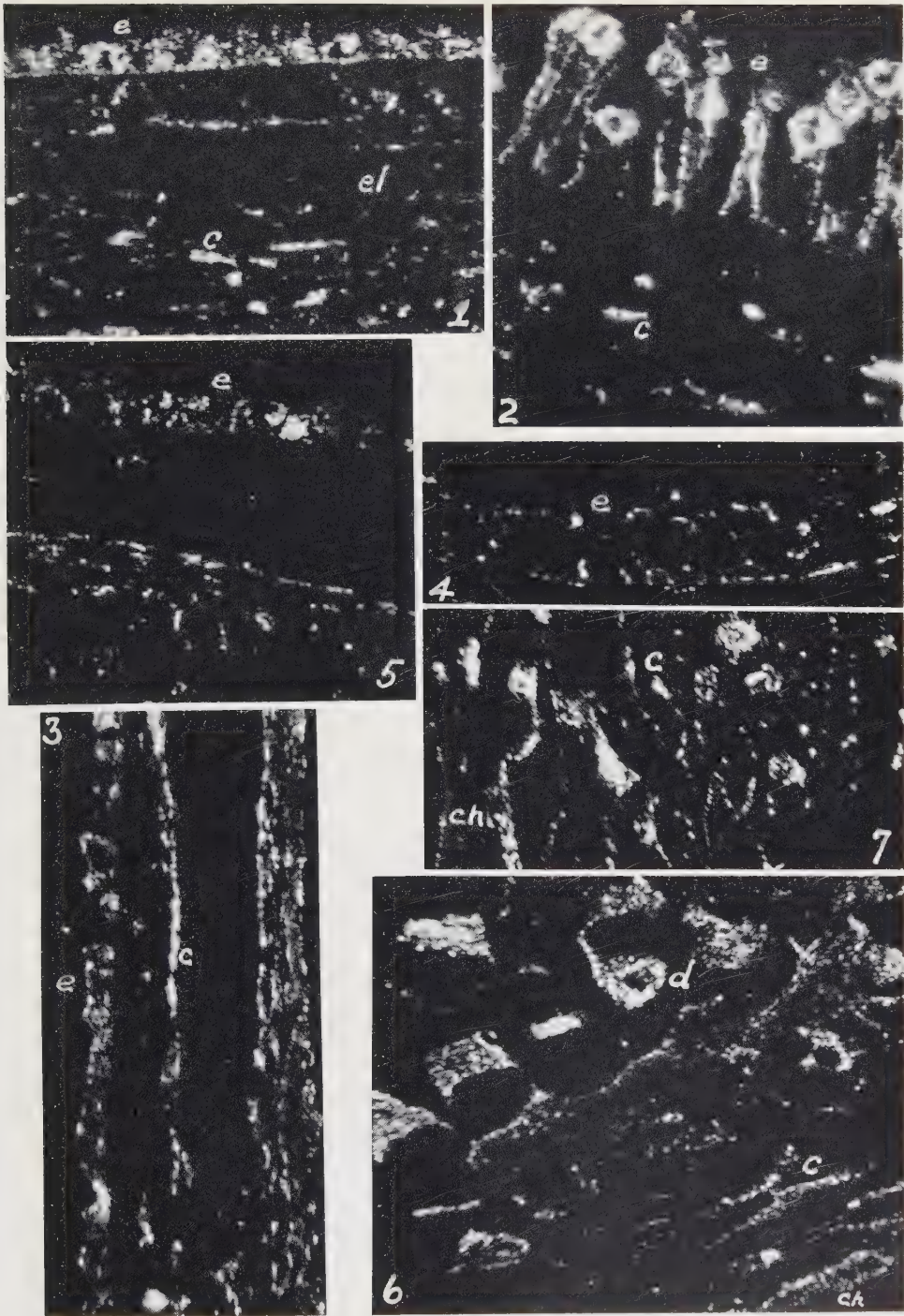
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PLATE 1

EXPLANATION OF FIGURES

e, epithelium of amnion	ch, chorionic epithelium
c, collagen	d, decidual cell
el, elastic fiber	

- 1 Free portion of amnion. $\times 610$.
- 2 Placental portion of amnion. $\times 650$.
- 3 Section similar to figure 1, showing loss of water-soluble ash. $\times 610$.
- 4 Section similar to figure 1 (epithelium only), showing non-acid soluble ash chiefly at base and top of cells. $\times 650$.
- 5 Same, but showing non-acid soluble ash scattered though chiefly at lateral cell boundaries. $\times 610$.
- 6 Chorionic epithelium, connective tissue and decidua. $\times 610$.
- 7 Same, showing distribution of non-acid soluble ash. $\times 610$.



THE COURSE OF THE BLOOD THROUGH THE FETAL HEART

AN EXPERIMENTAL STUDY IN THE CAT AND GUINEA PIG

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ONE FIGURE

It is doubtful if any subject in embryology has provoked as much discussion as the present one and still remained in a state of uncertainty. Whether there is a separation or a complete mixing of the superior and inferior vena caval blood streams in the fetal right atrium is a question which has been thrown back and forth by investigators for at least three centuries. William Harvey described the course of the fetal blood through the venae cavae, atria and foramen ovale; and before his time the attention of many others, including Vesalius and Galen, was directed to the structure of the fetal heart and the significance of its various openings and valves. A number of well written reviews of the subject are available. Those who are interested in its historical aspects may refer to Dalton's (1884) monograph and Pohlman's ('09) article.

One can not easily imagine what the state of physiological science would be if we had no more information about the adult circulation than we have about that in the fetus. Without exact knowledge of the course of blood in the fetal heart we are still in the seventeenth century, as it were, in respect to much of our information in fetal physiology. We still must speculate and make assumptions generously in many fundamental problems; indeed, there is scarcely any problem in

¹ Aided by a grant from the John and Mary R. Markle Foundation.

fetal physiology which is independent of circulation and respiration.

We propose not to review the literature on the subject of circulation through the fetal heart for we believe that it is well known by most investigators in the field of embryology. Furthermore, it has been adequately discussed in several key articles (Preyer, 1885; Pohlman, '09; Kellogg, '28, '30; Patten, '33; Barcroft, '36; Windle, '40). It is enough to say that methods of inductive and deductive reasoning on the basis of anatomical observations have guided (and misguided) many investigators, and that many of the analytical or experimental procedures introduced have been complex, technically difficult to use, and poorly controlled. We shall confine our attention in the present article largely to the results of a series of experiments by a very simple method, yet one which seems entirely adequate to give a true picture of the circulation in in fetuses brought into view by hysterotomy.

MATERIAL AND METHODS

We have used cat and guinea pig fetuses delivered with their placental circulations still intact under nembutal, local procaine infiltration of the abdominal wall, and without anesthesia but after decerebration of the mother by ligating the carotid and basilar arteries (Pollock and Davis, '24). The guinea pig fetuses varied between 40 days gestation and full term; the cat fetuses, between 54 and 60 days gestation. The gestation period lasts approximately 67 days in each species. About half of the experiments were conducted with the animals submerged in a bath of Locke's solution which was kept at body temperature.

Fetuses were anesthetized when delivered after administration of nembutal to the mother, and it was unnecessary to tie them down before operating upon them. The degree of anesthesia either corresponded to that of the mother or was greater in the fetus than in the mother. When no anesthetic

or only a local anesthetic was employed, the fetuses had to be confined by tying their limbs securely to a grid submerged in the saline bath; care was exercised to keep their heads beneath the surface of the fluid to prevent inflation of their lungs with air. Precautions were taken to maintain a freely flowing placental circulation, and this was accomplished in most of the specimens.

In one group of experiments the fetal carotid arteries and jugular veins were displayed by dissection, and the color of the blood within them was compared with that in the umbilical arteries and veins. In a few fetuses the thorax was opened quickly, and the beating heart studied for color differences on the two sides.

In another group of experiments the fetal thorax was opened in the following manner: The fetal skin and underlying soft tissues were incised in the mid-line from the rostral end of the thorax to a point a centimeter or less from the umbilical cord. The xiphoid process was obtained by dissection and lifted forward to allow the diaphragm to be incised. With two artery forceps (blunt mosquito points), a triangular piece of the anterior thoracic wall (apex at the rostral end of the chest) was grasped and excised. Thus the heart and lungs were displayed quickly and with negligible blood loss. Small quantities (from less than 0.1 cc. to 0.2 cc.) of black India ink were introduced into one umbilical vein through a fine calibre (no. 27) hypodermic needle. Precautions were usually taken to admit the ink gently into freely flowing blood. The passage of the ink into the heart and lungs could be observed directly. In some experiments it was timed with a stop-watch. Autopsies were performed at the conclusion of most of the experiments and histological sections of the lungs and heart were prepared. Fourteen guinea pig and fourteen cat fetuses were used.

Finally, a third group of experiments were performed in eight guinea pig and three cat fetuses. These were similar to

the former, but the ink was introduced into one of the external jugular veins (left in some; right in others).

The injections through the umbilical vein were so easy to perform and results were so striking that the method can be recommended to demonstrate the course of blood through the fetal heart before elementary students in embryology.

OBSERVATIONS AND RESULTS

When the blood coursing to the fetus in the umbilical vein had a bright red color and the umbilical arteries were dark, the carotid arteries appeared to be almost as red as the umbilical vein. Had there been complete mixing of blood in the right atrium of the fetal heart, one would have expected the carotid arteries to have been fully as dark as the umbilical arteries. Indeed, if there is a significant pulmonary venous return in the fetus, as other experiments indicate (Abel and Windle, '39), and if there is complete mixing of the caval streams, one should expect the carotid blood to be rather darker than that of the aorta and its branches. Such a condition was never encountered.

It was hoped that direct observation of the beating heart would yield information on the present subject. Another investigator had recorded color differences on the two sides of the heart of the fetal guinea pig (Barcroft, '36). When the umbilical vein blood was bright red and that of the umbilical arteries dull, we could see little or no difference in color of the two ventricles. Their walls were too thick. In the youngest fetuses the left ventricular apex was slightly brighter than the right. On the other hand, when we examined the auricles of the fetal heart a striking picture presented itself: the left was as brilliantly colored as the umbilical vein while the right was as dark as the jugular veins.

When 0.2 cc. of India ink was introduced into the umbilical vein its passage to the heart could be observed. Almost the first indication that it had reached the fetal heart was a sudden blackening of the entire ventricular wall. This was not due to a sudden filling of all four cardiac chambers. On

the contrary, critical observation revealed that the ink entered the left atrium and left ventricle first, and immediately afterward passed to the aorta and its coronary branches to darken the heart muscles. When the fetal heart was beating rapidly and vigorously, it took from 3.5 to 7.5 seconds for the ink to pass from a point in the umbilical vein about 2.5 cm. from the fetal abdomen to the coronary vessels. The color of the fetal lungs did not change at the time the heart darkened, and bits of tissue clipped from a lung at this time proved histologically to contain no carbon. A small pleural vessel was



Fig. 1 The thoracic viscera of two littermate cat fetuses of about 54 days gestation. India ink (0.1 cc. in the left and 0.2 cc. in the right) had been introduced into the umbilical veins. For explanation of the color differences in the lungs, see the text.

injected in one specimen. However, 13 to 17.5 seconds later the lungs did darken as illustrated on the right in figure 1. This interval represents the circulation time from the left ventricle to the periphery, back to the right side of the heart, and thence to pulmonary vessels. Sections of lung tissue removed at this time were full of carbon.

When 0.1 cc. of India ink was introduced into the umbilical vein the fetal heart blackened as before (within 10 seconds or less). But the fetal lungs did not darken at all. This is

illustrated by the specimen on the left in figure 1. Histologically, no carbon was found in the lung tissue. Examination of the fetal brain in these experiments demonstrated that the ink had reached it, for it appeared gray. The venous blood leaving the brain proved upon microscopic examination to contain only an occasional particle of the ink. The carbon had been deposited in the periphery and no excess was available to be carried back to the right side of the heart and to the lungs when 0.1 cc. was used instead of 0.2 cc.

Several attempts were made to inject very small amounts of ink into the umbilical vein before the chest was opened, and then to open the thorax before the ink could be flushed entirely out of the heart. We found some carbon in the coronary circulation but none in the lungs.

When about 0.1 cc. of India ink was introduced gently into either the left or right external jugular vein of the fetus, it traversed the superior vena cava and made its appearance in the right auricle. It did not enter the left auricle at this time, and the coronary circulation remained red. The ink could be observed to pass out of the right ventricle in the pulmonary trunk; within a second or two it appeared in the lungs. Much carbon was observed in sections of these organs. Only after the ink had darkened the lungs did the left auricle and the coronary vessels darken, and in no instance did they become as black as the right auricle and the pulmonary vessels.

One experiment in a cat fetus of about 54 days gestation was particularly striking. About 0.05 cc. of ink was introduced into the left external jugular vein. It appeared first in the right auricle, coloring this chamber black. Soon the lungs could be observed to darken slightly, but little ink returned from them to the left side of the heart. The coronary vessels and ventricular walls did not blacken, but a perceptible graying could be seen. We then introduced about 0.1 cc. of ink into the umbilical vein of this fetus. The left auricle blackened, and almost at the same instant we could see the ink course out over the coronary arteries, blackening both ventricles simultaneously.

The observations we have described up to this point pertain to fetuses in good health whose placental circulations were intact and whose hearts were beating vigorously. We have made observations on three guinea pigs and one cat fetus in which the heart beat had become feeble and the normal circulation was impaired. Ink injected into either vena cava in these specimens could be seen to enter both left and right atria slowly and at almost the same instant.

DISCUSSION

The present study proves that most of the blood of the inferior vena cava makes its way to the left side of the fetal heart, and that of the superior vena cava, to the right side with little or no mixing of the two streams taking place in the right atrium of the cat and guinea pig fetus under the experimental conditions prevailing. What were these conditions? The fetuses, some anesthetized and some unanesthetized, had been delivered by hysterotomy with placental circulation intact. The thorax was open in some, closed in others, and the heart was beating vigorously. The lungs had not filled with air. We can not say that conditions within the undisturbed uterus are entirely comparable with those of our experiments, but they are at least as good as the best of our experimental conditions. We found that the better the health of the fetus, and the more vigorous its heart beat, the more clearly demonstrable was the separation of the two blood streams.

Anatomically the fetal heart is so constructed that the streams will cross, providing that the blood is flowing under some pressure and the anatomical shunts are patent. When the heart slows and the pressure within it declines, ink introduced into either stream goes slowly both to the left and the right side of the heart. This is highly significant because the conditions of these few experiments are similar to those of the only other investigators who used living fetuses (pig) and obtained evidence that the two caval streams mix in the right atrium (Pohlman, '09; Kellogg, '28).

Perhaps there are species differences in respect to the course of blood through the fetal heart. Our experiments apply only to the cat and guinea pig. However, Kellogg ('28) obtained similar evidence in one litter of dog fetuses in which starch suspensions were injected into the umbilical vein under conditions similar to ours. Greater amounts of starch were recovered from the left ventricles than from the right. This is the more remarkable when consideration is given to the fact of pulmonary venous dilution of the left atrial contents (Abel and Windle, '39). Furthermore, Barclay and others ('39) have demonstrated very clearly in sheep fetuses delivered by hysterotomy that the superior caval stream is directed entirely to the right ventricle, and the inferior, largely through the foramen ovale to the left side of the heart and only slightly into the right.

In three widely differing classes of mammals (rodents, ungulates, carnivores) then, it has been experimentally demonstrated that the vena caval streams traverse the right atrium of the fetal heart with little or no admixture. We are unable to say whether other species including man are similar. Patten's ('33) careful investigations of the structure of the human fetal heart led him to favor the theory of mixing. In one living human fetus of 4 months gestation we opened the umbilical arteries and injected Müller fluid into the umbilical vein to fix the central nervous system *in situ*. The head and arms turned an orange color before we could see any change in the abdomen and legs. Of course this observation proves nothing, but it suggests that much of the inferior caval stream goes directly to the left side of the human fetal heart; carefully controlled experiments should be performed.

Little need be said concerning the significance of our present observations. Clearly, the rostral parts of the body are supplied with more highly oxygenated blood than the caudal parts. Barcroft and others ('40) have recently demonstrated that the carotid arterial blood of sheep fetuses (obtained without delivering them) is actually as much as 88% saturated with oxygen. Thus they have established that neither the

fetal brain nor the fetal heart, which incidently is the only organ working as hard in prenatal life as after birth, thrive on reduced blood in utero. The crossing of the caval blood streams makes adequate oxygenation of these organs possible. Any attempts to determine the physiological activities of the fetal central nervous system must take these facts into consideration.

CONCLUSIONS

During the last third of gestation in the cat and guinea pig fetus all or nearly all of the blood entering the heart by way of the inferior vena cava passes into the left atrium and left ventricle to be delivered to the heart muscle, brain, and upper parts of the fetal body.

All or nearly all of the blood from the superior vena cava traverses the right atrium and right ventricle from which it is sent to the fetal lungs and the lower parts of the body including the placenta.

Mixing of the two caval streams of blood in the right atrium was not demonstrable so long as the fetal circulation and heart action were in good physiological condition. But when the heart had ceased to beat vigorously and the fetus was in a dying state, the sluggish streams of blood did mix in the right atrium.

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THE TOPOGRAPHY OF THE HYPOPHYSIS IN THE ELEPHANT, MANATEE AND HYRAX

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ONE TEXT FIGURE AND THREE PLATES (FOURTEEN FIGURES)

The pituitary gland in these three orders of mammals is being compared because comparative anatomists regard these animals as being more closely related to one another than to the other groups of mammals. The elephant and manatee are large animals, the latter aquatic, whereas the hyracoids are small, far less specialized forms which by some are regarded as belonging to a proto-ungulate stock. The extremely close similarity in these three mammals in regard to placentation has recently been demonstrated by Wislocki ('35) and Wislocki and Westhuysen ('40).

MATERIAL

From the elephant (*Elephas indicus*) and from the Florida manatee (*Trichechus latirostris*) we have one hypophysis from each. Of hyrax (*Procavia capensis*) we possess three serially sectioned pituitaries. We are indebted to Mr. Dan Harkins, Director of the Zoological Garden of the City of Boston for the elephant specimen; to Mr. George Nelson of the Museum of Comparative Zoology at Harvard University for the manatee hypophysis; and to Messrs. O. P. van der Westhuysen and Roderick Murray for several specimens of *Procavia capensis* from South Africa. The elephant was known to be about 30 years old, whereas the manatee was a nearly grown animal. The specimens of *Procavia* were from adult animals. The material in all cases had been fixed in 10% formalin. The preservation is good, with the exception

of the elephant in which 18 hours elapsed after death before the hypophysis was placed in fixative. The majority of the sections were stained in hematoxylin and eosin. In the case of the manatee, resort was had also to sections stained with Mallory's connective tissue stain, while sections of the elephant's hypophysis were stained for iron (Prussian blue reaction) and calcium (von Kossa's method). For the latter preparations we are indebted to Dr. Sidney Farber, Pathologist at the Children's Hospital, Boston. The pituitary gland was weighed only in the case of the elephant; in the manatee and hyrax the hypophysis was left attached to the brain, a circumstance which precluded obtaining its weight.

Previous mention or descriptions of the hypophysis of these animals exists, to our knowledge, only for the elephant (Stendell, '14) and the manatee (Oldham, McCleery and Geiling, '38 and Geiling, '40).

INDIAN ELEPHANT

The hypophysis of this specimen was described and illustrated in a previous paper (Wislocki, '39). In summary it may be stated that the pituitary gland of the 30-year old adult animal weighs 7.5 gms. and consists of a large anterior lobe (6.7 gms.), a small neural lobe (0.8 gm.), and a pars tuberalis which clasps the neural stalk. The anterior lobe and the neural lobe are connected by a layer of connective tissue which separates the specific elements of these two parts. A pars intermedia is not demonstrable. The anterior lobe is composed of epithelial cords separated in places from one another by an excessive amount of connective tissue. Numerous small colloid follicles occur throughout the anterior lobe, some of which have undergone calcification.

Since the account of this hypophysis was published, our attention has been directed to a previous description of the elephant's pituitary. In Stendell's monograph on the pituitary gland ('14), he gives a fragmentary account, with only one figure, of the hypophysis of an elephant which he mentions as having been very old. His description differs in

one major particular from ours in that he devotes several paragraphs to the description of a well-developed pars intermedia. Re-examination of our specimen fails to confirm Stendell in this regard, and leads to an inquiry as to whether his observation and ours can be in any way reconciled. It should be recalled that pars intermedia was a term introduced by Herring in 1908 to define the entire juxtaneural portion of the glandular pituitary, but that it was not until 1913 at the recommendation of Tilney that the pars tuberalis was subtracted from the pars intermedia. Since Stendell makes no sharp discrimination between pars tuberalis and pars intermedia, it is quite probable that he included all or part of the present-day pars tuberalis within his definition of pars intermedia. In our specimen a pars tuberalis is easily discernible and cords of tuberalis cells extend for a short distance between the anterior lobe and neural lobe; this latter part Stendell may have regarded as being pars intermedia. He states further that the pars intermedia contains small colloid-bearing follicles and he uses the presence of these follicles as a criterion for appraising the extent and size of the intermedia. In our own specimen, on the contrary, small colloid follicles are widely scattered throughout the entire anterior lobe and do not lend themselves for purposes of discriminating between a pars distalis and a pars intermedia. In our preparations typical cords of pars distalis cells appear to extend up to the zone of connective tissue separating the anterior and posterior lobes.

Before leaving the elephant's pituitary, we regard it as necessary to introduce some additional observations regarding the nature of the small follicles scattered throughout the anterior lobe. The majority of these follicles contain colloid which may be diffuse or arranged in rings. The colloid is acidophilic. In occasional follicles the colloid appears to be coagulated in the form of an irregular network. The majority of the follicles are enclosed by epithelial cells, although in some the epithelium appears to be degenerating or to have

disappeared. In the latter case the drop of colloid is encapsulated by connective tissue.

The colloid in many of these follicles reacts positively for iron (Prussian blue reaction). The iron reaction may occur diffusely throughout the follicle, or may be limited mainly to its periphery, including the cells and connective tissue surrounding it.

A minority of the follicles react strongly for calcium (von Kossa's reaction). The calcium may occur in the form of a single central spherule, or mass of minute spherules. It may also be present as a peripheral shell of calcium with a hollow center, or, finally, as a succession of calcareous rings, comparable to the concentric rings of colloid observed in some of the non-calcified follicles. The interior of the follicles, which contain calcium, stains deep blue with hematoxylin in contrast to the affinity for eosin shown by the colloid in the ordinary follicles. The epithelial cells around the calcified spherules are occasionally totally degenerated. Within some of the hollow calcified follicles the centrally situated colloid takes the form of a stringy network.

These observations regarding the follicles are presented in some detail because Stendell ('14) makes no mention of either iron or calcium in the pituitary of the Indian elephant examined by him. Furthermore, he attributes the presence of the colloid follicles, which he observed, to the extreme old age of his animal. The hypophysis of our elephant at thirty years contains an abundance of follicles in its anterior lobe, a change which may be referable to aging even though the animal cannot be regarded as senescent. Our findings are recorded also because of the occurrence of concretions in adenomas of the human pituitary and of the speculations as to their genesis advanced by Lisco ('37).

FLORIDA MANATEE (*TRICHECHUS LATIROSTRIS*)

The gross appearance of the pituitary of the Florida manatee is shown in figure 1. It consists of anterior and neural lobes which are both well developed. The anterior lobe

(p. d.) forms a thick curved mass applied to the antero-ventral surface of the large neural lobe (i.p.). Rounded lateral projections of the anterior lobe flank the sides of the medially situated neural lobe. The dorsal and posterior surfaces of the infundibular process are not surrounded by the epithelial hypophysis; consequently the distal end of the



Fig. 1. Drawing of the hypophysis and hypothalamic region of the Florida manatee, cut in the midsagittal plane ($\times 8$). p. d., pars distalis; i. p., infundibular process; m. e., median eminence; c. m., mammillary body. Observe the nodular median eminence. The small drawing ($\times 3$) inserted at the lower left, shows the hypophysis in situ when viewed from the ventral surface. Observe how the anterior lobe clasps the neural lobe leaving the pole of the infundibular process completely exposed. Compare these drawings with an actual midsagittal section of the pituitary in figure 2.

infundibular process forms a conspicuous protuberance extending posteriorly beyond the borders of the epithelial lobe.

The short infundibular stalk attaches broadly to the tuber cinereum. The third ventricle forms a very shallow trough-shaped recess in the proximal part of the stalk. The walls of this recess are formed by that portion of the stalk designated as the median eminence (fig. 1, m.e.). Unlike other mammals, the ventricular surface of the shallow infundibular recess is thrown into a succession or corona of small nodular protuberances.

A series of sagittal low power views of the hypophysis are presented in figures 2, 3 and 4. These reveal the anterior lobe, the relatively large infundibular process, the short, broad infundibular stalk, and the above mentioned low tubercles of the median eminence.

It should be noted also that between the anterior lobe and the neurohypophysis there is an extensive series of clefts containing eosin staining colloid besides some cellular material. To what extent these clefts represent a complete system of intercommunicating diverticula, or are separated from one another, has not been systematically investigated. Several smaller spaces have been traced sufficiently to establish that they do not communicate with the larger ones.

A pars tuberalis is present but in very attenuated form. It is scarcely visible in the low power photographs (figs. 2 and 4, p. t.). It is identifiable on the anterior and lateral surfaces of the median eminence, but appears to be absent on the posterior surface of the stalk. Figures 7 and 10 present two views of the tissue composing the pars tuberalis; figure 10 is from the anterior border of the median eminence in the mid-sagittal plane, whereas figure 7 is located more laterally. Both of the photographs illustrate well the character of the pars tuberalis. It is relatively slight in amount and extent, and consists of small, deeply staining cells which form clusters in the neighborhood of, or actually line, a series of slightly dilated, minute clefts or spaces. The small tuberalis cells lining these clefts appear to be inactive and none of them are

ciliated. No colloid or stainable secretion is visible in the lumen.

The pars intermedia, like the pars tuberalis, appears to be relatively attenuated. Figures 5, 6 and 9 are taken at various levels in the midline along the border between the anterior and posterior lobes. The photographs show portions of the extensive residual lumen. The residual clefts are lined by a distinct epithelium of variable height ranging from active, tall, columnar cells to flattened, inactive ones. Anteriorly the clefts are flanked by typical anterior lobe tissue. Posteriorly, abutting on the infundibular process, the epithelial tissue is relatively scant. In places the clefts are separated from the infundibular process by a single row of columnar or flattened cells lining the residual lumen. In some places, however, posterior to the residual lumen, the cells are more plentiful and become aggregated into small masses which are irregularly attached to the neural lobe. Such clusters of cells are present particularly on the lateral surfaces of the infundibular stem and process. These cells have the appearance of typical pars intermedia, consisting of clusters of medium-sized cells which stain moderately deeply with basic dyes.

The cells constituting the intermedia and bordering the residual lumen about the infundibular process but do not come into direct contact with the elements of the latter, for a very definite lamina of collagenous connective tissue separates the two and clothes the infundibular process. This narrow, somewhat irregular layer of connective tissue separating the pars intermedia from the infundibular process is clearly visible in figures 5 and 6 which are from sections stained with Mallory's connective tissue stain. The layer is somewhat disrupted or irregular because in places pars intermedia cells appear to have invaded it. In no areas, however, do the epithelial cells appear to have completely broken through this connective tissue barrier to penetrate actually the specific neural lobe tissue. Consequently we have in our specimen of the Florida manatee a condition in which, although an attenuated pars

intermedia is present, it is separated nevertheless from the neural tissues by a distinct connective tissue barrier.

In regard to the South American manatee (*Trichechus inunguis*), Oldham, McCleery and Geiling ('38) have described a different situation. They state that in that species the gland consists of two lobes separated by a thick layer of dense connective tissue. They conclude furthermore that gross or microscopic evidence of an intermediate lobe is lacking.

More recently Geiling ('40) mentions briefly the results of examination of a Florida manatee. Of this animal he remarks that the cleft in the posterior region of the anterior lobe is even more prominent than in the South American manatee and that the posterior border is formed of several layers of cells. Furthermore, the separation between the glandular and neural lobes is not complete, for ventrally the cells forming the posterior border of the cleft appear to be intimately associated with the neural lobe and even invade it. These cells, Geiling states, may represent the intermediate lobe. Our own more extensive study of the histology of the Florida manatee's hypophysis leads us to the conclusion that this animal possesses a true, although attenuated, *pars intermedia* which is separated, however, in our specimen, from the specific neural tissue by a very definite lamina of connective tissue which invests the neural stem and process.

The *pars distalis* in our specimen has not been stained in a way that yields any important information regarding its cytology. The presence of a few small follicles with open centers surrounded by epithelial cells should be recorded. Some of these are spherical, whereas others form short clefts. They occur here and there in all parts of the *pars distalis*, although the largest, most elongated ones appear to be in the zone of the *pars distalis* bordering the *pars tuberalis*. These follicular and tubular structures contain a variable amount of stainable colloid or stringy material. The cells lining them may be either cuboidal or columnar. When cuboidal, the nuclei are situated basally with an outer lavender cytoplasm bordering the lumen. In some follicles, particularly those in

the transitional zone between the pars distalis and the tuberalis, the columnar cells are very conspicuously ciliated. In the smaller follicles deeply situated in the anterior lobe the cells lining them are mostly small and cuboidal and take a pale basic stain. In occasional follicles typical acidophiles and basophiles line the lumen in place of the above mentioned smaller cuboidal cells.

The neural lobe and stalk warrant brief comment. The former is quite vascular. The blood vessels are accompanied by delicate sheaths of reticular fibers as demonstrated by connective tissue stains. The vessels are so arranged that the tissue of the neural lobe is divided up into a curious mosaic. The neural stalk is also quite vascular, but here the vessels are arranged differently. Arterioles and venules penetrate from the surface of the stalk into the interior in the form of isolated vascular tufts which in cross section give the appearance shown in figure 8. These tufts consist of a core in which an arteriole is often observed accompanied by a number of satellite capillaries, the whole vascular core being imbedded in a sheath of interweaving collagenous fibers and attendant cells. Around this core the neural tissue is arranged radially, consisting of an almost acellular field of fibrils. Outside this acellular coronal zone in the interstices between the vascular tufts, lanes of relatively cellular neural tissue are present. The club-shaped ends of such vascular tufts with the enclosing tissue produce the low protuberances which are observed projecting into the interior of the ventricle in the region of the median eminence.

HYRAX (*PROCAVIA CAPENSIS*)

The hypophysis of this animal can be more briefly described. It consists of a well-defined pars distalis, pars tuberalis, pars intermedia and neurohypophysis (figs. 12, 13, 14 and 15). The gland lies long drawn out beneath the diencephalon and mid-brain in a shallow trough formed by the sphenoid bone. The anterior lobe lies beneath the elongated neurohypophysis, enfolding the neural stalk laterally without, however, completely

encircling it. The pars tuberalis extends anteriorly from the pars distalis, forming a delicate mantle completely encircling the median eminence (fig. 14).

The pars intermedia is well developed and extensive (figs. 11, 12 and 13, p. i.). Ventrally it is separated by a long but extremely narrow, and hence inconspicuous, cleft from the pars distalis. Anteriorly, where the residual lumen ceases, it merges with elements of the pars distalis and pars tuberalis in a manner which obscures the exact location of its boundary. Laterally it envelopes the neural lobe without, however, completely enclosing it. Posteriorly it extends to the tip of the infundibular process and in one instance forms a cap situated over the terminal pole of the neural lobe (fig. 15).

The residual lumen is exceedingly narrow, contains a small amount of colloid, and is lined by small cuboidal cells (fig. 11).

The cells of the pars intermedia are relatively uniform, small, basophilic elements which are arranged in a distinctly tubular or alveolar manner (fig. 11). The tubes and alveoli enclose irregular-shaped lumina which contain pale, pink-staining secretion.

From the third ventricle a slender, narrow recess extends into the neural stalk terminating at the beginning of the solid infundibular process. Thus the infundibular recess, although narrow, extends about one-third of the length of the neurohypophysis. At the point of communication of the recess with the third ventricle there appears to be a definite constriction of the lumen.

There are several accessory but rather novel features of the hypophysis of *Procavia*. One is the presence (fig. 15) of a well-developed, persistent craniopharyngeal canal through the sphenoid bone into which a prolongation of the pars distalis extends. In a second specimen a similar although lesser canal is present with a small, sequestered, cystic mass of distalis cells within it.

In the specimen shown in figure 15 the dural sellar diaphragm has imbedded in it two isolated nodules of bone.

Thus the pituitary of *Procavia*, unlike either the elephant or the manatee, has all parts of the pituitary well represented, including a well-developed *pars intermedia* as well as a *pars tuberalis*.

DISCUSSION

In respect to ample representation of both *pars tuberalis* and *pars intermedia*, *Procavia* resembles the majority of mammals and in this regard is more primitive than either manatee or elephant in which these parts are either reduced or absent. Recent investigations have revealed several other mammals in which these portions of the pituitary are rudimentary or lacking (Wislocki and Geiling, '36; Wislocki, '38). Below a summary is presented of these recent observations:

Pars tuberalis: Reduced: Manatee

Absent: Sloth, Anteater

Pars intermedia: Reduced: Manatee

Absent: Elephant, Cetacea, Armadillo

Reduction of *pars tuberalis* and *pars intermedia* is evidently an evolutionary step occurring in only a few species of mammals. It is doubtful whether these more unstable parts of the gland possess as much functional importance as the anterior and neural lobes which are invariably present in all species, although the neural lobe may, as in the whale and elephant, be relatively small.

In those species possessing little or no *pars tuberalis* or *pars intermedia*, no common factor can be discerned. Of the group listed above only the elephants, whales, and manatees are excessively large animals, whereas only the whales and manatees are aquatic mammals. That the modifications of the pituitary in the whales and manatees are not an obligatory result of adaptation to an aquatic mode of life is indicated by the fact that two aquatic members of the carnivores—seal and otter—which we have examined, have a well-developed *pars tuberalis* and *pars intermedia* and resemble closely other carnivores in regard to the topography of their pituitaries.

The facts emerge, however, from these various observations that in certain mammals neither the pars intermedia nor the pars tuberalis is absolutely essential and that the neural lobe may be separated from the pars intermedia or anterior lobe by a connective tissue barrier. In the presence of the latter condition there appears to be little possibility for direct interchange of hormonal substances between the two main parts of the gland. In such species, for example, the whales, Geiling ('40) has successfully assayed the two separate portions of the gland for their respective hormones.

SUMMARY

The hypophysis of a 30-year old Indian elephant weighs 7.5 gm. and consists of a large anterior lobe (6.7 gm.), a small neural lobe (0.8 gm.), and a pars tuberalis which clasps the neural stalk. A pars intermedia is not demonstrable. Anterior and neural lobes are separated by a stout sheet of connective tissue. The anterior lobe contains numerous minute colloid follicles, some of which are calcified.

The hypophysis of a nearly grown Florida manatee has well-developed anterior and posterior lobes. The pars tuberalis and pars intermedia are present but very much reduced. A distinct lamina of connective tissue intervenes between the pars intermedia and the neural stalk and lobe.

The hypophysis of adult specimens of *Procavia capensis* (Hyrax) possesses a well-differentiated pars tuberalis and an extensive pars intermedia, besides a large anterior lobe and a well-developed infundibular stalk and process.

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PLATE 1

EXPLANATION OF FIGURES

2 Midsagittal section of the pituitary of the manatee. Observe the large anterior and neural lobes, the short infundibular stalk, the nodular median eminence, the attenuated pars tuberalis (p.t) and the extensive residual lumen. $\times 11$.

3 The same pituitary in a paramedian plane. Extensive clefts filled with colloid can be seen in the posterior part of the anterior lobe. These extend laterally on the sides of the neural stalk. $\times 11$.

4 A section of the same pituitary situated still more laterally. Notice the extensive residual lumen. Between this lumen and the neural lobe there is a narrow pars intermedia. Observe also the pars tuberalis (p.t.). $\times 11$.

5 A portion of the residual lumen, flanked anteriorly (left) by pars distalis, and bordered posteriorly by one or more rows and small clusters of cells constituting the pars intermedia. Between the epithelium of the pars intermedia and the infundibular process (right) there is a lamina of connective tissue stained deeply by Mallory's connective tissue stain. $\times 90$.

6 Another portion of the residual lumen, bordered posteriorly by an attenuated pars intermedia. The sheet of connective tissue separating the epithelial hypophysis from the neural lobe is again clearly shown. Mallory's connective tissue stain. $\times 80$.

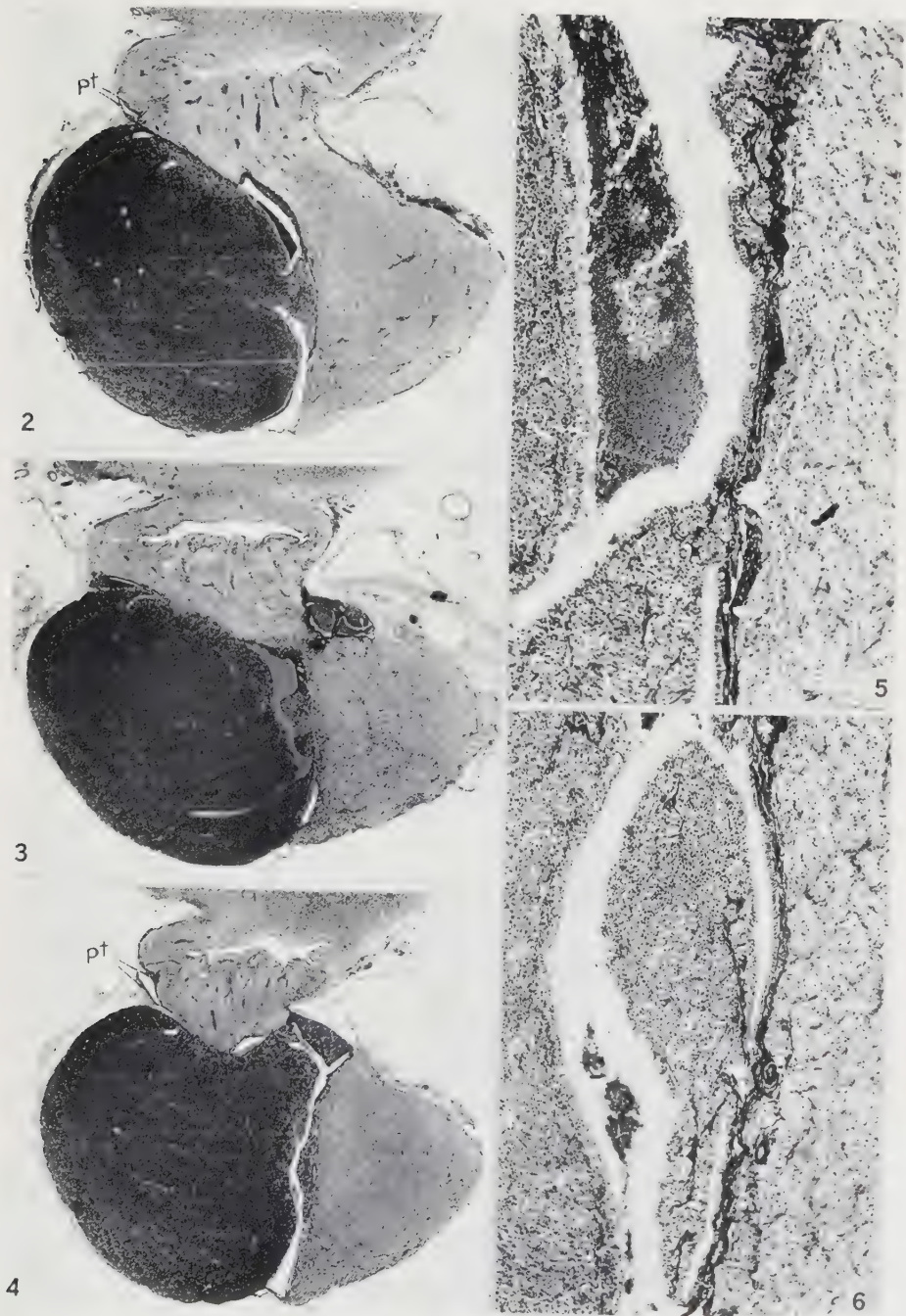


PLATE 2

EXPLANATION OF FIGURES

7 A portion of the relatively attenuated pars tuberalis showing the characteristic clefts lined by epithelium. On the right the tissue of the infundibular stalk is visible. $\times 90$.

8 A portion of the median eminence of the neural stalk, showing several of the vascular tufts characteristic of this region. The tuft seen in the upper part of the photograph has been cut transversely and shows the succession of circular layers described in some detail in the text. $\times 97$.

9 The residual lumen is shown with the pars distalis on the left and on the right an elongated mass of epithelium posterior to the lumen, between it and the infundibular process. In this section, stained with hematoxylin and eosin, the connective tissue lamina encapsulating the neural lobe is not visible. $\times 80$.

10 Typical pars tuberalis consisting of a cleft surrounded by nests of epithelial cells. On the right the neural stalk is visible, while on the left the leptomeninges are situated. In figure 2 the small cleft in the upper left hand corner of the hypophysis, on the surface of the infundibular stalk and just above the border of the anterior lobe, is the same one shown in this photograph. $\times 10$.

11 The hypophysis of *Procavia capensis*, showing the well-developed pars intermedia (p.i.) in the middle, separated from the anterior lobe (p.d.) on the left by an extremely narrow residual lumen, and abutting the neural lobe (i.p.) on the right. Notice that the cells of the pars intermedia tend to be arranged in clusters with hollow centers. $\times 80$.

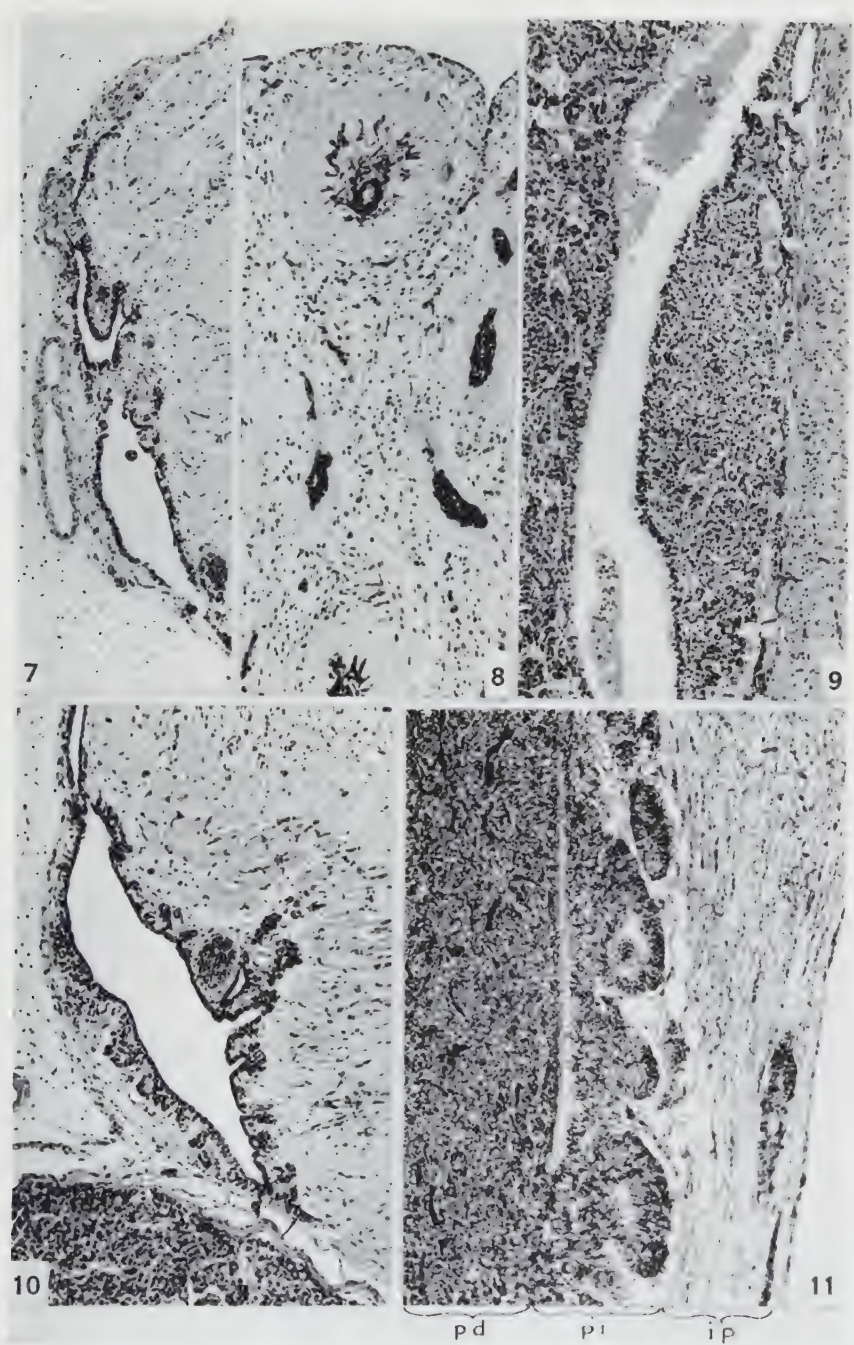


PLATE 3

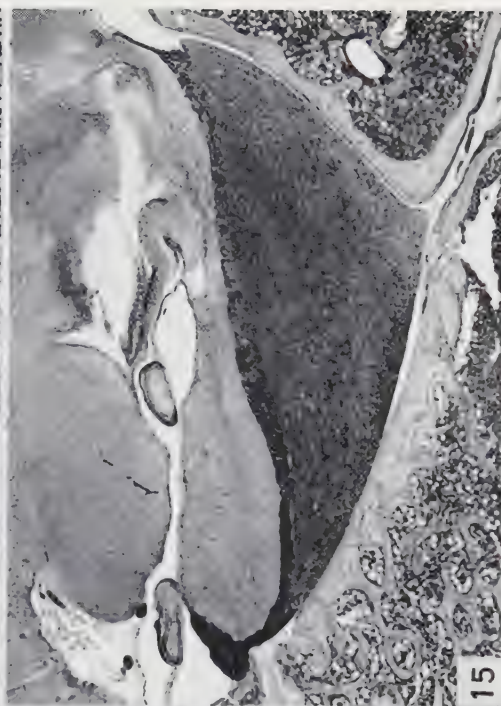
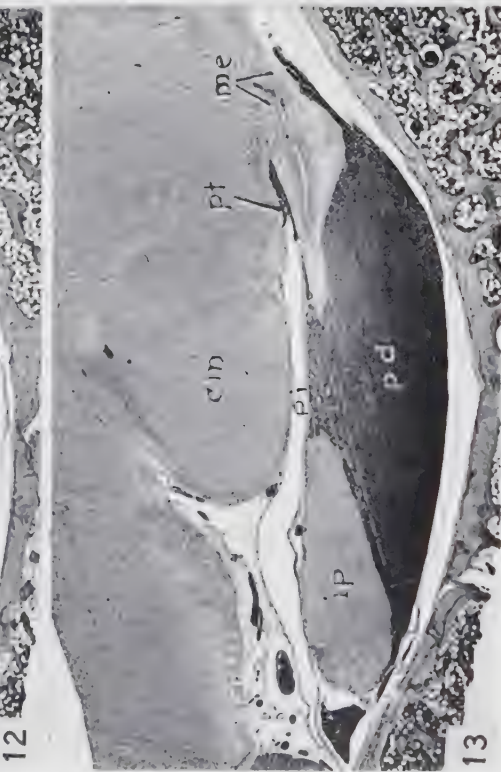
EXPLANATION OF FIGURES

12 Low power view of the hypophysis of *Procavia capensis* cut in the mid-sagittal plane. Observe the presence of an anterior lobe, neural lobe, pars intermedia, pars tuberalis and a narrow infundibular recess extending from the third ventricle for some distance into the neural stalk. The residual lumen is so narrow that it does not effectively set off the dark pars intermedia from the anterior lobe at this magnification. $\times 10$.

13 A paramedian section of the same hypophysis. The delicately lobulated and extensive pars intermedia (p.i.) is seen to better advantage in this plane. The pars tuberalis (p.t.) is also clearly visible on the anterior and posterior borders of the infundibular stalk, forming small black bands of tissue. I.p., infundibular process; m.e., median eminence; p.d., pars distalis.

14 A section of the hypophysis taken farther laterally. The pars intermedia partially envelopes the infundibular process. The pars tuberalis is visible as a deeply stained sheet of tissue bordering the infundibular stalk. $\times 10$.

15 Another pituitary of *Procavia capensis*, showing a well-developed craniopharyngeal canal traversing the sphenoid bone. In this specimen the pars intermedia is very well differentiated and extends posteriorly to form a deeply staining mass of tissue covering the posterior pole of the infundibular process. In the sellar diaphragm above the body of the pituitary two ovoid masses of bone are visible in the dura. $\times 10$.



THE BLOOD VESSELS OF THE ADRENAL GLAND OF THE ADULT CAT ¹

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THREE TEXT FIGURES AND FOUR PLATES (TWENTY FIGURES)

INTRODUCTION

This paper deals with the blood supply to the adrenals of the cat, the distribution of blood vessels within the gland, and the relationship between the blood vessels and the cells of the cortex and medulla. A number of anatomical features of the blood vessels of the adrenal which have not been noted by previous authors are presented, and many of Flint's ('00) findings in the dog are confirmed in the case of the cat. This paper is illustrated by accurate drawings and by photographs, instead of by somewhat schematized drawings such as Flint used to document his paper.

MATERIALS AND METHODS

Cats were used throughout. Preparations of several types were made as follows:

1. *Preparations for dissection.* Colored injection masses were prepared by mixing sifted red lead, lithopone, or Prussian blue with a warm gelatin solution, or by grinding an oil suspension of artist's oil colors in thickened methyl methacrylate according to a modification of Schummer's injection method ('35). Methyl methacrylate was found to be a suitable medium

¹ This work was supported in part by a grant from the Josiah Macy, Jr. Foundation.

² Fellow of the National Research Council (1937-1939) when most of the work for this paper was performed.

for introducing fine pigment into vessels. After injecting contrasting pigments into the arteries and veins of the adrenal through the abdominal aorta and vena cava respectively, the material was fixed in formalin and the retroperitoneal tissue dissected out in a block which contained the adrenals, kidneys, inferior portions of the diaphragm, and surrounding tissues including portions of the aorta and vena cava. The block was dehydrated and cleared in benzene and oil of wintergreen according to the Spalteholz method, and the blood vessels dissected out under a binocular microscope.

2. *Preparations for sectioning.* Adrenals were injected with the above-mentioned pigments if the larger arterioles and venules were to be visualized, or with diluted India ink or 4% Berlin blue if the capillaries and smaller vessels were to be demonstrated. The glands were then removed and fixed. Some were cleared by the Spalteholz method and studied whole and after freehand sectioning. Others were embedded in celloidin or in amyl acetate-nitrocellulose after the author's ('40 a) method and sectioned serially and mounted on slides. Some glands were cut into thick sections (100–350 μ) and mounted unstained. Others were cut into thinner sections (10–100 μ) and stained lightly with hematoxylin and eosin before mounting. In a few instances alternating thick and thin sections were cut and the thin sections stained and mounted separately, according to the technique used in a former study on the blood vessels of the embryo pig heart (Bennett, '36).

3. *Casts.* Methyl methacrylate was injected into the vessels of the adrenal and polymerized in situ, according to a modification of Schummer's ('35) method of making vascular casts. The methyl methacrylate was injected dyed or colorless, or mixed with coarse or fine pigments, and frequently the veins and arteries were injected in contrasting colors. In no instance were perfect casts of the entire adrenal capillary bed obtained, but the imperfect casts were a great aid in obtaining a three-dimensional picture of the vasculature of the organ.

OBSERVATIONS

1. Main arterial supply and venous drainage

The sources of the arteries supplying the adrenals are best seen in dissections of injected and cleared blocks of the retroperitoneal region surrounding the adrenal glands. In the specimen illustrated in figure 1, the blood supply of the adrenal can be seen as numerous small arteries arising from most of the large trunks in the vicinity, including the aorta, both renal arteries, the adrenolumbar arteries, one ileolumbar artery, the phrenic arteries, and the coeliac axis. There is

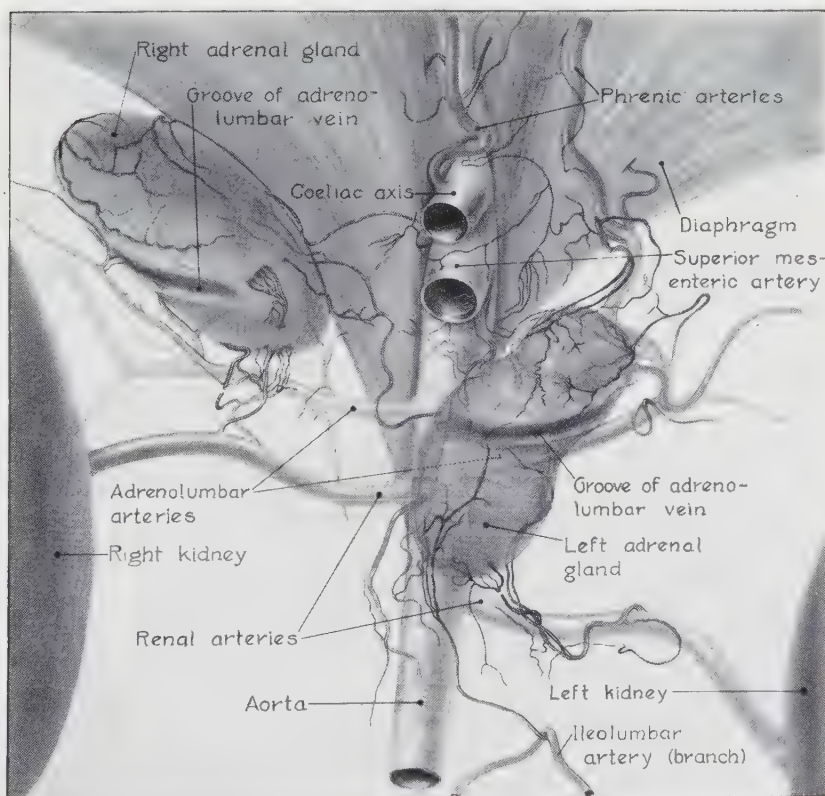


Fig. 1 A dissection of an injected and cleared block of retroperitoneal tissue in the region of the adrenal gland. The arteries have been filled with finely ground chrome yellow in thickened methyl methacrylate. All veins have been dissected away.

considerable variation in the exact origins and the pathways of the adrenal arteries from cat to cat, and no one arrangement can be considered typical. It is not unusual for arteries to arise from the superior mesenteric artery and pass to the adrenal, though no such artery can be demonstrated in the specimen illustrated. The origin of the cat's numerous adrenal arteries from all or most of the big arterial trunks passing close to the gland is much as described by Flint ('00) in the dog, and by von Ebner ('02), Gérard ('13), and by Anson, Pick and Beaton ('39) in the human. Most of these workers have also noted a variability in the adrenal arterial pattern in the dog and in the human similar to that seen in the cat, and it appears that in none of these three forms is one justified in describing the arterial supply to the adrenal as consisting of a superior, a middle, and an inferior adrenal artery, as is commonly stated in the current textbooks of human anatomy.

The venous drainage of the cat's adrenal is much more constant. The adrenolumbar vein in the cat usually passes directly over the gland, making a groove across the adrenal. Occasionally this large vein may actually tunnel through a portion of the gland. The medullary veins of the adrenal gland proper open directly through several small apertures into this adrenolumbar vein as it passes over or through the gland. In rare instances the adrenolumbar vein does not come into direct contact with the gland, but passes by the gland a few millimeters away from it through the retroperitoneal connective tissue. In such cases one can see two to four slender veins draining the adrenal and bridging the space between the gland and the adrenolumbar vein, into which they empty.

The adrenal veins of the cat's medulla have no demonstrable muscle fibers in their walls. Bargmann ('33) lists the cat among the animals without a rich musculature in the medullary veins. This is contrary to the situation in the human, where Ferguson ('06), Kolmer ('18), and Bargmann ('33) have noted longitudinal smooth muscle in the walls of the central adrenal vein and some of its larger branches.

Kutschera-Aichbergen ('22) has described anastomoses between the capsular veins of the adrenal and the branches of the hepatic portal vein in the human. A careful search of numerous well-injected preparations has failed to reveal any such anastomoses in the cat, where, indeed, "capsular" veins are of trifling size and number.

2. *Finer distribution of the arteries and veins*

Upon more detailed examination of cleared injected blocks of tissue containing the adrenal glands similar to that shown in figure 1, one can see that the numerous arteries passing to the adrenal gland branch and spray out shortly before they reach the surface of the organ (figs. 1 and 2). Not infrequently small branches of the arteries anastomose directly with tiny venous radicals connected with the veins draining the tissues around the gland (fig. 2). Four or five of these arteriovenous anastomoses can be seen in the connective tissue around a single adrenal.

The branching arteries pass to the adrenal, where they abruptly penetrate the capsule and assume a position directly under the connective tissue of the capsule and outside the parenchymal cells of the gland proper (figs. 6 and 15). Under the capsule these arteries ramify and anastomose freely, forming an extensive subcapsular arterial plexus which invests the gland, and whence vessels are given off which supply the entire cortex (fig. 3). At fairly regular intervals arteries leave the subcapsular plexus at right angles and pass radially through the cortex to the medulla. These are the arteriae medullae described by Flint ('00), and their subsequent course and ultimate distribution will be described later in this paper.

A curious feature of the subcapsular plexus of the cat which appears to have escaped the notice of previous investigators is the fact that the arteries running under the capsule frequently dip down into the cortical parenchyma and then return to the subcapsular region, forming arterial loops (figs. 3, 6, 8 and 9). The arteries forming these loops or recurrent dips return to the capsule without branching as a rule (figs. 8 and 9),

although exceptionally one of the radial medullary arteries may spring from the curve of such a loop and pass centripetally to the medulla (fig. 6).

The arteries of the subcapsular plexus branch repeatedly and form a multitude of fine twigs (fig. 4) which give rise to

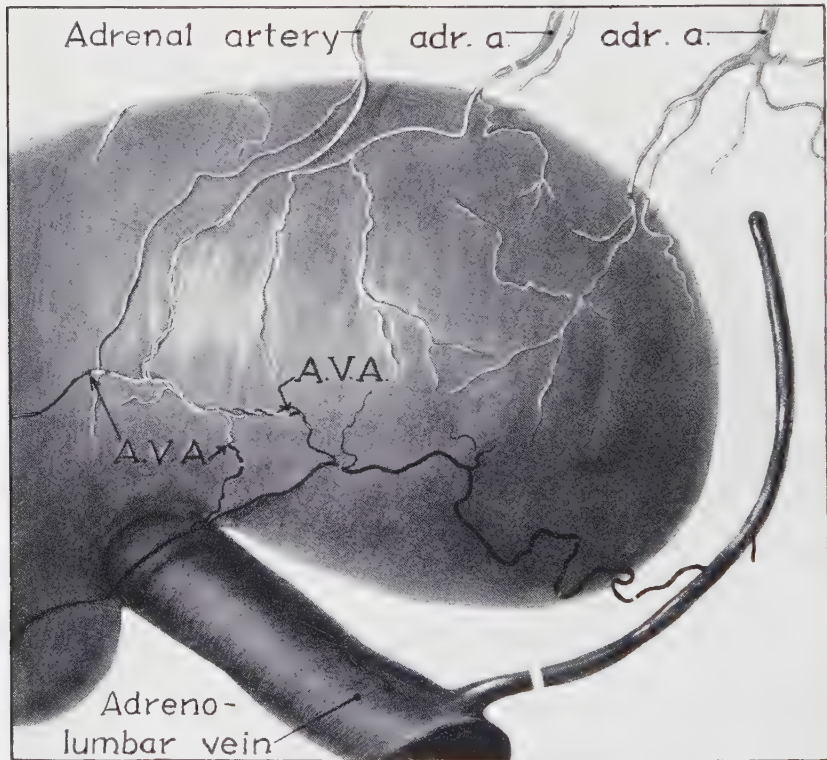


Fig. 2 Dissection of a cleared block of tissue surrounding an adrenal gland. Arteries light, veins dark. The breaks in the continuity of the vessels represents places where the vessels were inadvertently broken in dissection. Note the arterio-venous anastomoses (A.V.A.).

a subcapsular capillary plexus. This capillary plexus lies in the same plane as does the arterial plexus, and when injected it is seen on a surface view as a rich and extensive anastomosing capillary network which overlies the coils or knots of

cells which form the zona glomerulosa of the cortex (fig. 5). It is from this subcapsular plexus that the capillary bed of the adrenal cortex flows.

In the cat the only vessels within the cortex itself are capillaries, with the exception of the arteriae medullae and the

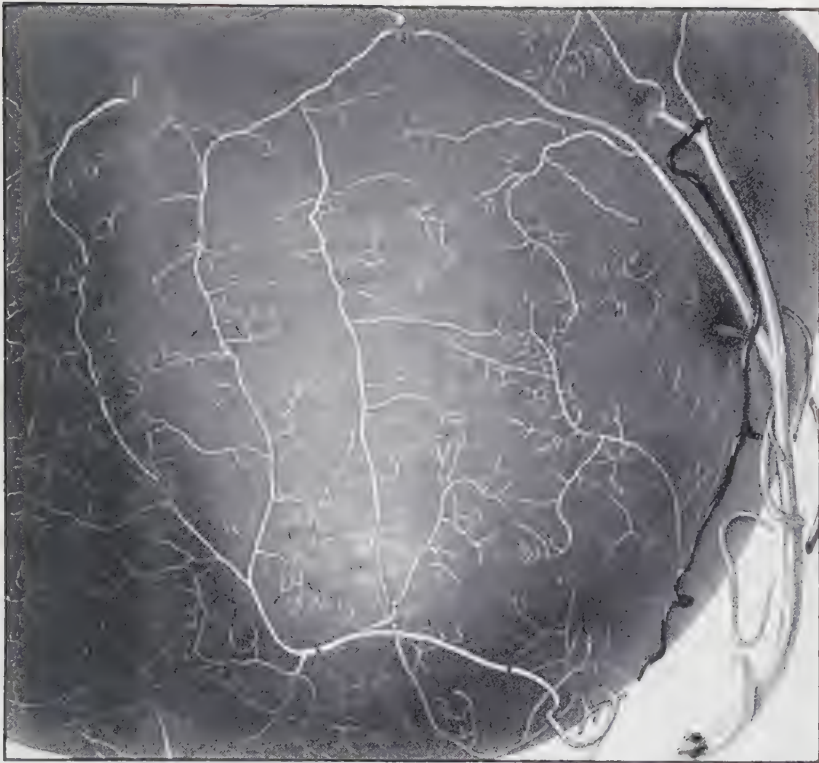


Fig. 3 A surface view of an adrenal with small arteries injected, showing the anastomosing ramifications of the subcapsular arterial plexus. On the large arterial loop passing upward on the left one can see three places where the artery dips down deep into the cortical tissue and then returns to continue on in the subcapsular region, forming the recurrent loops mentioned in the text.

recurrent arterial loops mentioned above. The “arteriae corticis” which Flint ('00) describes as penetrating the cortex of the dog to break up into cortical capillaries cannot be seen in the cat. Similarly, we have been unable to find any “arteriae

capsulae," described by Flint as nourishing the capsule. In the cat, the surface plexus of arteries and capillaries is predominantly subcapsular and is not embedded in the capsule as in the dog (Flint, '00). Consequently one cannot separate out two groups of vessels, one of which nourishes the capsule and the other the cortex. Moreover, the arterial loops described above do not break up into capillaries, and hence cannot be considered as equivalent to the "arteriae corticis" of Flint.

3. The capillaries of the cortex

The subcapsular capillary plexus should be considered as part of the capillary supply to the cortex, because its meshes abut directly on the parenchyma of the glomerulosa. From this plexus innumerable radial cortical capillaries are given off at right angles and pass centripetally toward the medulla. These radial cortical capillaries branch off and turn into the cortex in the depressions and grooves between adjacent knots of glomerulosa cells (fig. 10). Thus each knot or coil in the glomerulosa is embraced by an incomplete annular crown of capillaries. In addition a series of radial capillaries pass on the several sides of the coil toward the medulla in the delicate connective tissue septa which separate the groups of glomerulosa cells. This arrangement is much like that in the dog, as described by Flint ('00), and in thick sections of injected glands the location and outline of the glomerulosa arcades are clearly outlined by the capillaries surrounding them (figs. 13 and 14). These capillaries follow the tortuous borders of the coils until the straight columns of the zona fasciculata are reached. Here the capillaries become straighter and run parallel to the columns toward the medulla (figs. 13 and 14), taking positions so that each column of cells may be in contact with two to five capillaries. There is a certain amount of branching and anastomosing of the capillaries in the fasciculata. The capillaries in the outer portion of this zone are narrower than the capillary segments of the inner part of the zone (fig. 13). Frequently specimens show that material injected into the arteries has failed to pass through the con-

stricted segments into the outer portion of the fasciculata. Conversely, in figures 7, 11 and 12, one can see sections of glands where a mass injected retrogradely into the veins has filled the wide inner cortical capillaries but has failed to pass the barrier of the narrow vessels of the outer fasciculata. These constricted vessels on the outer fasciculata characterize the area thought to be the secretory zone of the cortex, where the cells are so distended with lipoid that they compress the capillaries surrounding them (Bennett, '40 b).

The radial capillaries of the cortex widen perceptibly as they continue on into the inner portion of the fasciculata. This widening of the capillaries takes place as the cells of the secretory zone lose most of their lipoid and become markedly shrunken in the postsecretory zone (Bennett, '40 b) (fig. 13). The extent of the widening of the capillaries as they enter the postsecretory zone varies considerably from cat to cat, and is in some cases so slight as to be scarcely noticeable (fig. 14).

The capillaries continue their branching and anastomosing course toward the medulla, following the configuration of the cell columns. When the straight columns of the fasciculata become buckled and broken to form the reticularis, the capillary pattern also becomes more plexiform and intricate.

As noted in the previous paper ('40 b), there is a marked variation in the width and complexity of the reticular zone in individual cats, and the configuration of the blood vessels in the juxtamedullary region of the cortex reflects this variability. It is also pointed out in that paper that there are variations in the zoning of the cat's adrenal which can be related to age and sex. Here again the blood vessels of the cortex reflect this variation, particularly with regard to the stretch of narrow capillaries characterizing the secretory zone, and the point at which the capillaries widen at the outer border of the postsecretory zone. Throughout the cortex of the cat the capillaries are exceedingly thin-walled, apparently consisting solely of a wall of thin endothelium invested in a delicate wide-meshed network of argyrophile connective tissue fibers (Bennett, '40 b).

It should be noted that the direction of blood flow in the capillaries of the cortex is always parallel to and in the direction of the supposed course of migration of the parenchymal cells of the cortex from their formation and maturation in the outer zones to their senescence and death near the medulla (Bennett, '40 b).

4. Irregularities in the blood vessels of the cortex

In addition to the variations in zoning from gland to gland mentioned above, the cat frequently shows nodules and septa and other irregularities which may break up and distort the usual concentric pattern of the gland. These irregularities have been previously described (Bennett, '40 b), and in the present injected material it is apparent that they may markedly modify the vasculature of the gland as well as the arrangement of the cells. However, in all such irregularities it can be seen that the subcapsular plexus of blood vessels follows the folding and reflexions of the capsule, and that the capillaries arising from this plexus conform in such instances to the distorted course of the cell columns.

5. The venous drainage of the cortex

The vast majority of the cortical capillaries penetrate to the corticomedullary border and then pass across into the medulla. Immediately upon entering the medulla the vessels change markedly in character (fig. 12). At the corticomedullary boundary the capillaries coming from the cortex gather into venous branches which have two to five times the diameter of the cortical capillaries (fig. 12), as described by Landau ('08). These venous channels are radicals of the medullary veins, and form part of an extensive and highly branched venous tree which extends throughout the entire medulla (fig. 11). Consequently very nearly all of the blood which has nourished the cortex subsequently traverses the medulla through this venous tree.

The small fraction of cortical venous blood which does not traverse the medulla may escape by either of two types of channels:

The more common of these two pathways is by means of cortical capillaries which empty directly into the larger branches of the medullary veins in cases where such branches pass through cortical tissue on their way to the main adrenolumbar vein. Naturally blood escaping through such channels does not come in contact with any medullary cells.

Less commonly one can demonstrate tiny surface veins which drain small groups of superficial cortical capillaries through the capsule and carry the blood to one of the numerous connective tissue veins located in the neighborhood of the adrenal. The venous drainage of surface nodules is usually of this type (fig. 7).

6. The blood vessels of the medulla

The medulla of the cat's adrenal, in agreement with Flint's ('00) findings in the dog, receives a twofold blood supply: one consisting of venous blood which has passed through the cortical capillaries into the medullary veins, and the other of blood which has been conveyed directly to the medulla by means of the radial medullary arteries. In the cat numerous medullary arteries (figs. 15 and 17) can be seen in sections of injected glands, and the vast majority of them proceed without branching until they have reached the medullary tissue. Occasionally such arteries may divide dichotomously while still in the cortex, but in such cases the two branches pass on to the medulla before breaking up into capillaries. These arteries do not give off branches which connect with cortical capillaries.

In the cat many of the medullary arteries show a considerable dilatation and tortuosity near the corticomedullary boundary (figs. 15, 16 and 17). This tortuosity usually becomes evident shortly before the artery reaches the medullary border, and characterizes the vessel well into the medulla. The tortuosity is often definitely spiral in character.

These arteries are thick walled and well supplied with muscle (Bennett, '40 b). It is evident from their histological structure and their size that they play no role in nourishing the cortex, and that they can supply the medulla with an abundant flow of arterial blood.

Upon reaching the medulla these arteries break up into an extensive bed of narrow, thin-walled capillaries which eventually drain into the medullary veins (figs. 16, 17, 18 and 19). The arteries are true end arteries, and do not anastomose with each other except through the capillary bed (fig. 16). Careful and painstaking search has not revealed a single instance where these medullary arteries anastomose with veins through channels other than the capillaries. Each artery appears to have a fairly definite group of medullary cells to supply, and oftentimes injected specimens show beds of medullary capillaries supplied by separate arteries (figs. 16, 18 and 19).

7. The precise relationship between the medullary blood vessels and the chromaffin cells

From figure 11 it can be clearly seen that the adrenal veins and their radicals, formed by the junction of cortical capillaries, penetrate every portion of the adrenal medulla, and figure 17 makes it equally evident that all parts of the medulla are nourished by means of capillaries supplied with blood through the medullary arteries described above. Careful examination of figures 17, 20 and 21 will show that the medullary arteries and their branches take positions between the ramifications of the venous tree. In stained, injected specimens where the cells have been lightly tinged, it is apparent that the ramifications of the capillaries are, in general, separated from the veins by the length of a single medullary cell. In consequence of this the chromaffin cells along the veins present, in general, one pole to the vein, while the other end of the cell is close to a capillary receiving fresh blood from one of the medullary arteries (figs. 22 and 23). Thus the medullary cells possess a true polarity with respect to the blood vessels, displaying a venous pole, which is perhaps the predominantly

secretory pole of the cell, and a capillary pole, which is presumably nutrient.³

This essential relationship between the cells and the blood vessels of the medulla can be seen readily in the medulla of properly injected glands whenever the plane of section cutting a vein is favorable. The relationships are particularly evident when the vessel is cut transversely (fig. 22), or longitudinally through a plane passing through or near the maximum diameter of the vein (fig. 23). In medullary cells cut in planes other than those parallel to the long axis of the cell their polarity to the vessels is not apparent. Similarly, this polarity is less evident in the cells around the smallest terminal venous twigs.

From a careful study of many sections it appears probable that a minority of the medullary cells fail to abut directly on the wall of a vein, whereas a larger fraction—possibly a majority—of the cells do not directly contact a capillary. But even these cells which do not impinge directly on a vein or on a capillary may show the typical polarity of the medullary cells in that one end can usually be shown to be close to a vein, whereas the other end will not be far from an arterial capillary.

DISCUSSION

As pointed out in the foregoing description, the basic features of the blood vessels of the cat's adrenal are similar to those in the dog, described by Flint ('00), or in the human, as cited by von Ebner ('02).

The arteriovenous anastomoses noted in the loose connective tissue surrounding the adrenal have apparently not been observed before, and their physiological significance is conjectural. They do provide channels whereby blood which has entered an artery destined to supply the adrenal can escape into the adrenolumbar vein without passing through the adrenal vascular bed.

³ A preliminary note covering this point was presented by the senior author before the American Association of Anatomists at the Boston meetings in 1939 (*Anat. Rec.*, vol. 73, supplement, pp. 6-7).

The curious recurrent loops into the cortex formed so frequently by the subcapsular arteries are met with in numbers in every well-injected cat's adrenal, and one can only speculate as to their role. It is perhaps possible that they provide a slack to be taken up by the arteries whenever the adrenal gland increases in size as a result of vasodilatation or congestion of the many blood vessels within it.

A similar explanation would provide a logical *raison d'être* for the tortuosities that the medullary arteries undergo as they pass the corticomedullary boundary, although one must entertain the possibility that they may be fixation artefacts. These spiral twistings remind one of similar, though more complex, configurations noted in the arteries of the uterus (Daron, '36), and might very well provide for a ready lengthening of the artery whenever the gland expands with engorgement. No ready physiological explanation is apparent for the dilatation so frequently met with along the tortuous portions of these arteries.

It is perhaps appropriate to consider briefly the physiological significance of the relationships between the secreting cells of the adrenal and the blood vessels. In the case of the cortex each secreting cell is in contact with one to four very thin-walled capillaries whose endothelial lining is separated from the parenchymal cells only by a delicate network of argyrophile connective tissue reticulum (Flint, 1899; Bennett, '40 b). The blood flowing through all the capillaries passing a secreting cortical cell must be under approximately the same pressure and laden with equal amounts of oxygen and nutrient material. Hence these capillaries must function equally in nourishing the cell (except for difference in area of contact with individual capillaries and the cell), and it is clear that the self-same capillaries which nourish the cell must also carry away the secretion of the cell. This situation is seen in other endocrine organs, as in the corpus luteum, Graafian follicle, testes, and thyroid, in each of which the capillaries nourishing the cells probably also serve as the main channels for carrying away the secretion. In many of these organs the lymphatics

may dispense part of the secretion, although in the adrenal they appear to be unimportant.

The medulla, however, differs markedly from the cortex with respect to its vascularization. Here the secreting cells characteristically border on a venous radical at one pole of the cell, whereas the other pole is directed toward a capillary. This concept was clearly set forth by Hultgren and Andersson (1899), though they failed to present any evidence for their statement.

The medullary veins contain blood which has passed through the cortical capillaries and is hence depleted of much of its oxygen and nutrient material, whereas the capillaries are well supplied with fresh arterial blood at a hydrostatic pressure higher than that in the veins. Thus it would be logical to assume that the capillary supplies the cell with most of its metabolic raw materials, and the pole of the cell directed toward the capillary could be regarded as the "nutrient" pole of the cell. Evidence will be presented in a later paper to support the view that the venous pole of the cell is the surface through which most of the secretion escapes, and that it can be logically termed the "secretory" pole of the cell. Cytological data will also be given in that paper to support the idea of a polarity of the medullary cells.

The cells of the presecretory zone of the adrenal cortex may show a polarity with respect to the Golgi apparatus and the nearest capillary (Hoerr, '31; Bennett, '40 b), but these cells apparently are not actively secreting, and the polarity is lost when the cells reach the secretory phase.

Among other endocrine glands a polarity of the cells is obviously present in the thyroid, where one end of the cell presents to blood vessels and the other to the lumen of the follicle. A polarity based on cytological features has been noted in the hypophysis (Dawson and Friedgood, '37). A similar polarity has been alleged in the parathyroid (Courrier and Reiss, '22; Rosof, '34), although Cowdry ('22) failed to find a consistent polarity in the cells of this gland. Courrier and Reiss ('22) and Rosof ('34) regard the cells of the parathyroid as possessing a "nutrient" and an "excretory" capillary, but they fail

to present injection preparations or convincing evidence for this concept. These authors do not state whether the alleged two types of capillaries are distinct entities, whether they receive their blood from distinct types of vessels, or whether they contain blood in different states of oxygenation. Hence the cells of the adrenal medulla must be regarded as the only endocrine cells known to display a polarity with respect to two different types of blood vessels, and where the predominant nutrient function of a vessel at one pole of the cell and the secretory function of the vessel at the opposite pole can be inferred from injected preparations.

SUMMARY

The blood supply to the adrenal gland of the cat is described, and is found to be similar to that of the dog, as described by Flint ('00).

Features characterizing the blood supply to the adrenal which were not noted by Flint in the dog are as follows:

1. True arteriovenous anastomoses are found in the loose connective tissue surrounding the adrenal glands of the cat.

2. The subcapsular arteries of the cat's adrenal frequently dip down into the cortex proper and then return to the subcapsular region, forming numerous recurrent loops.

3. The penetrating radial medullary arteries usually show a characteristic dilatation and tortuosity—often spiral—as they pass the region of the corticomedullary border.

4. The arterially supplied capillaries of the medulla, and the medullary veins formed by the junction of the cortical capillaries, are so distributed as to give a double vascularization to all parts of the medulla. The vessels are arranged so that the cells of the medulla are characteristically endowed with a true polarity, with one pole of the cell directed toward or bordering on a vein, and the other end of the cell lying close to an arterially supplied capillary.

ACKNOWLEDGMENT

The authors wish to thank Dr. George B. Wislocki, under whose direction this work was performed, for his help and criticism in this investigation.

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PLATE 1

EXPLANATION OF FIGURES

4 Surface view of an adrenal injected with India ink. An artery can be seen approaching the surface and breaking up into the subcapsular plexus of arteries. $\times 25$.

5 Surface view of an adrenal injected with India ink. In this specimen the subcapsular plexus of capillaries has been filled in addition to the arteries, and the surface view of the capillary mosaic around the knots of glomerular cells is apparent. $\times 25$.

6 Section of gland injected with India ink. An artery can be seen approaching the gland, penetrating the capsule, and running over the surface of the cortex in the usual subcapsular position. It forms a recurrent dip into the cortex, and from the trough of the dip an artery can be seen branching off to pass radially to the medulla. $\times 25$.

7 Section of injected blood vessels around a surface nodule of cortical tissue. Note how the subcapsular arteries follow the connective tissue separating nodule from cortex proper, and then leave it to penetrate radially toward the medulla. The irregular stellate configuration on the side of the nodule away from the cortex represents ink in the veins draining the nodule. $\times 25$.

8 Section of injected gland, showing subcapsular arteries dipping down into the cortex and returning to the subcapsular layer, forming the recurrent arterial loops described in the text. The capillaries are not injected. $\times 58$.

9 Section of injected gland, showing a similar recurrent dip or loop of a subcapsular artery against a background of injected capillaries. $\times 58$.

10 Thin, stained section of injected adrenal, showing how the subcapsular capillaries embrace the glomerulosal knots and send branches about them radially toward the medulla. $\times 150$.

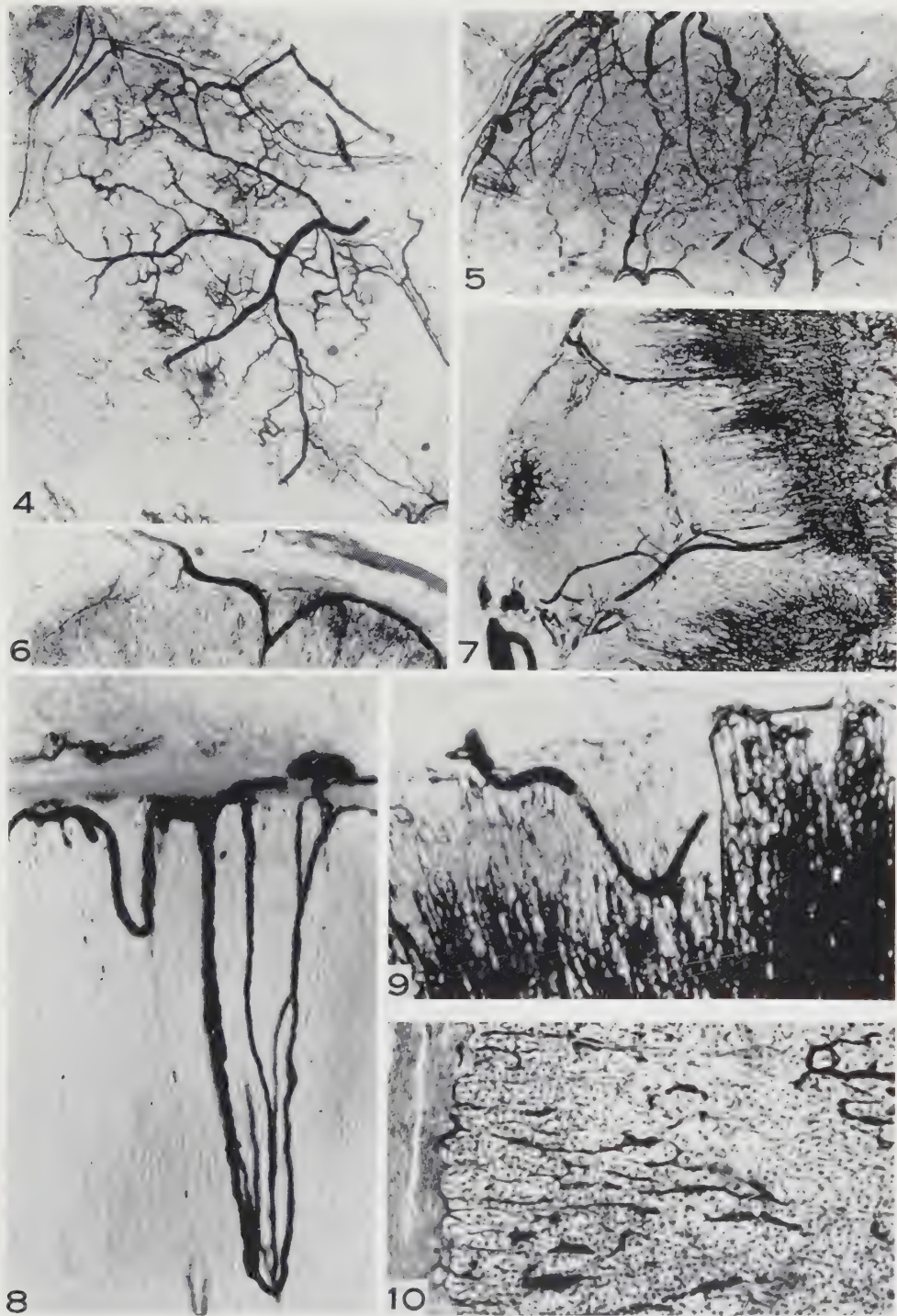


PLATE 2

EXPLANATION OF FIGURES

11 Section of adrenal gland injected through the veins with Berlin blue gelatin. The large black mass in the lower right corner is part of the adrenolumbar vein. Two large medullary veins can be seen emptying into it, and the smaller venous radicals can be seen reaching all parts of the medulla. Some of the medullary capillaries have been filled by retrograde injection. $\times 25$.

12 Section of adrenal gland injected through the veins with Berlin blue gelatin. Corticomedullary boundary. Note the character of the transition between the cortical capillaries streaming from above and gathering into the terminal radicals of the medullary veins. $\times 58$.

13 A virtually complete India ink injection of cortical capillaries. The black mass on the lower left is ink in the adrenolumbar vein. The capsule dipping down into the groove of the vein, and the subcapsular capillary plexus can be seen following this configuration. The capillaries can be seen outlining the glomerulosa arcades, and then paralleling the cell columns of the fasciculata toward the medulla. The capillaries are narrow in the outer portion of the fasciculata, in the region of the secretory zone. $\times 58$.

14 A similar preparation, showing a connective tissue strand penetrating the cortex and distorting it, carrying a double layer of capillaries with it. The continuity between the capillary layers along the strand and the subcapsular capillaries along the outer surface of the cortex is obvious. In the cortex proper, the vessels clearly outline the configuration of the cortical cells. $\times 58$.

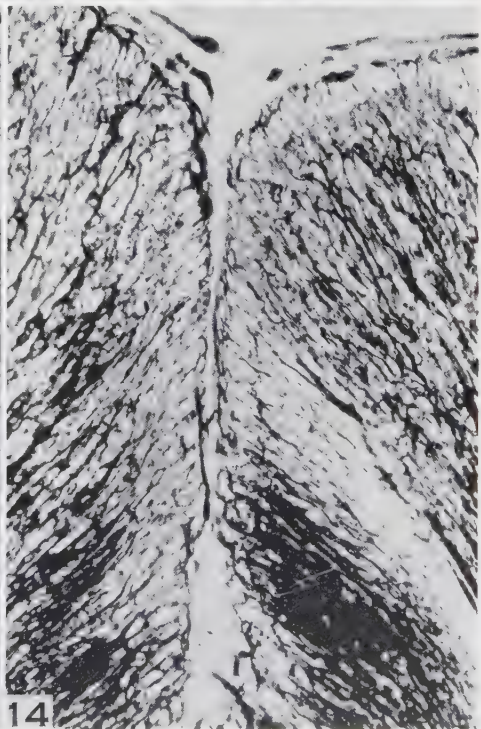


PLATE 3

EXPLANATION OF FIGURES

15 Section of injected gland including part of the medulla, cortex and surrounding loose connective tissue. Several arteries can be seen approaching the gland and penetrating the capsule. One can be clearly seen to turn at right angles and pass radially toward the medulla. The tortuosity and dilatation characteristic of medullary arteries as they pass the corticomedullary border are apparent in this instance. The capillary bed is rather patchily injected. $\times 25$.

16 Section of injected gland, showing two medullary arteries entering the medulla and breaking up into their respective, though anastomosing, capillary beds. Both arteries show typical tortuosities. $\times 58$.

17 Section through cortex and medulla of injected gland. Several arteries can be seen approaching the adrenal through the surrounding tissue, and forming the subcapsular plexus. Several medullary arteries can be seen, some with tortuosities. The medullary venous tree and capillary bed are injected, and the veins can be seen passing toward the adrenolumbar vein, which is represented by the black mass on the left. $\times 13$.

18 and 19 Terminal branches of two medullary arteries showing the fineness of the capillaries which they supply. $\times 58$.

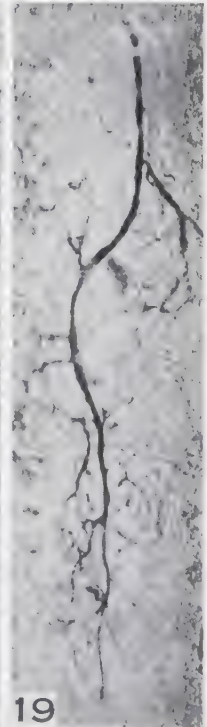
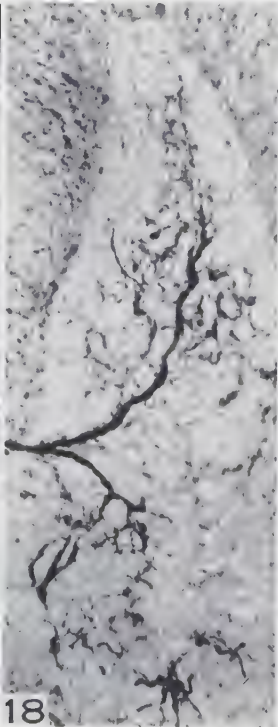
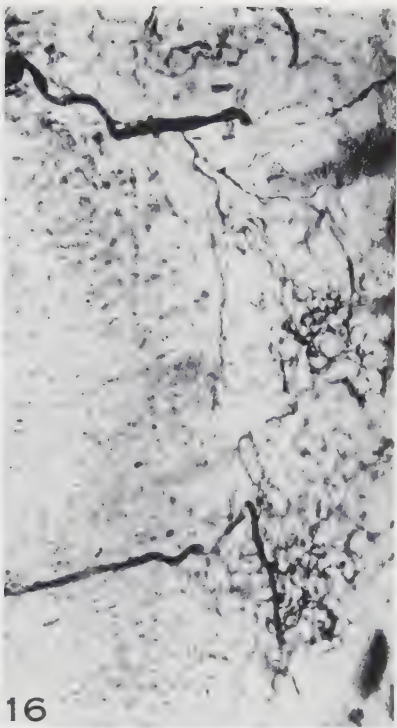
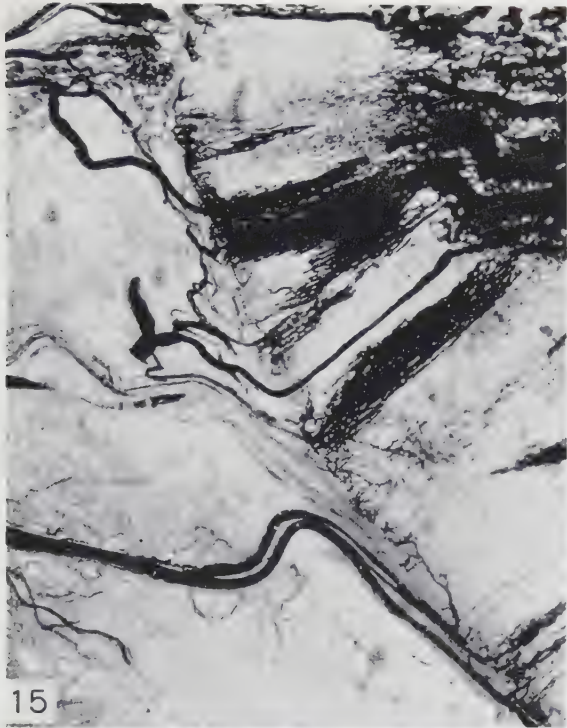


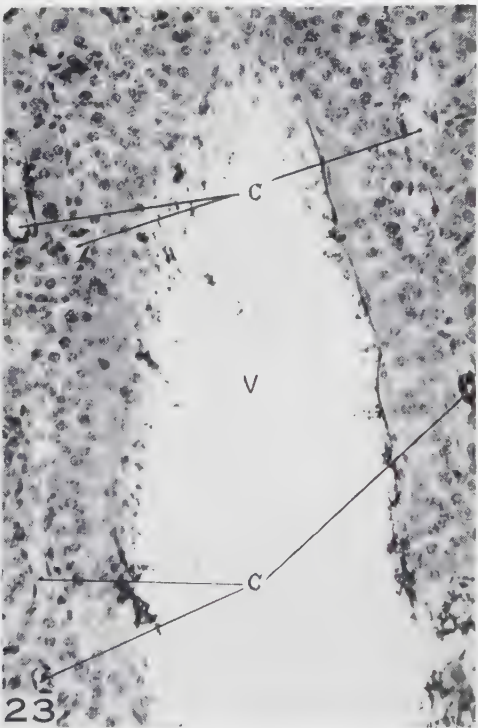
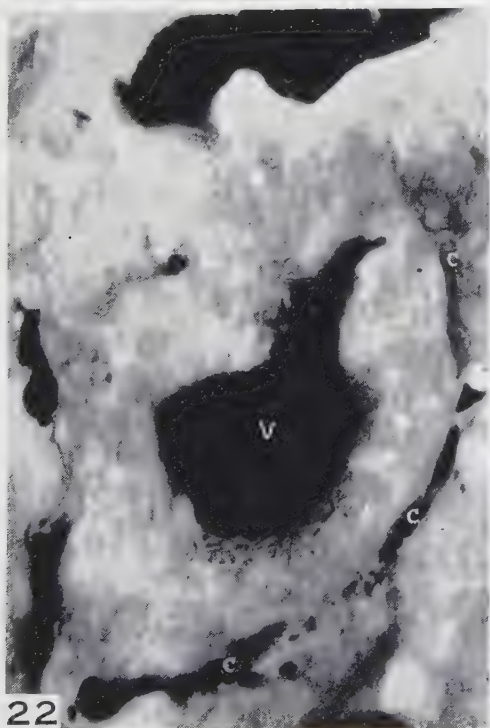
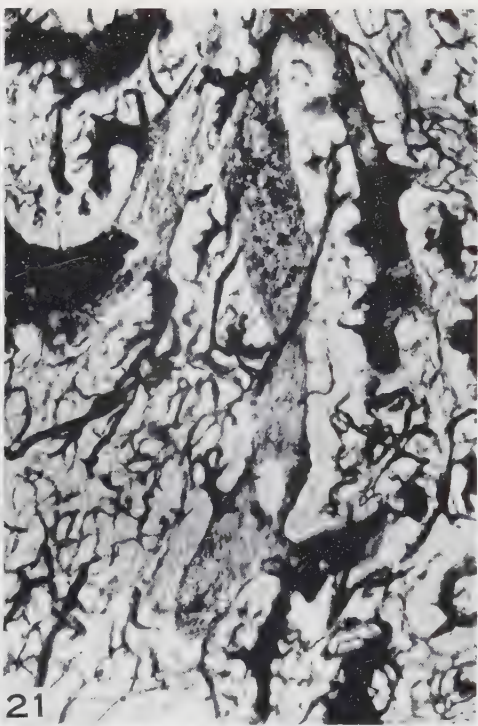
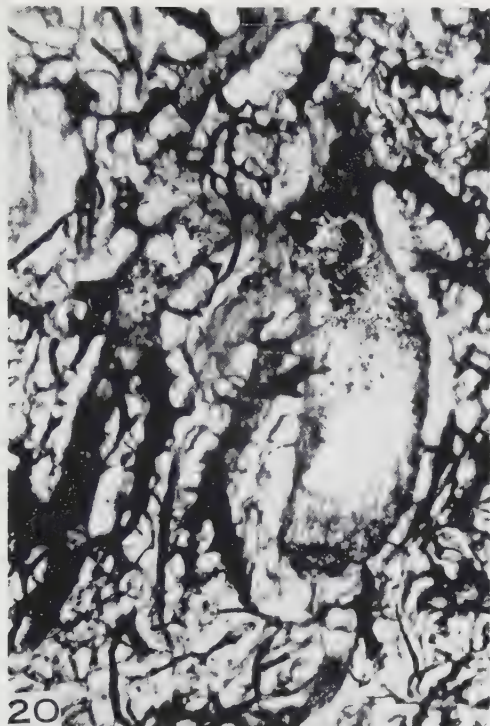
PLATE 4

EXPLANATION OF FIGURES

20 and 21 Sections of injected adrenal medulla. The large, branching structures are veins, and between them are arterially supplied capillaries which drain into the veins. $\times 75$.

22 Stained section of an injected adrenal medulla. Field represents the cross section of a vein (V), with a surrounding rosette of medullary cells bordering on it. The other pole of many of the cells is closely applied to arterially supplied capillaries (C). $\times 430$.

23 Another preparation to show the vascular polarity of the cells. The large vein (V) is seen to be bordered by a columnar layer of cells, which present at the other end toward capillaries (C), which are in turn supplied by arteries. $\times 200$.



A HISTOLOGICAL STUDY OF FAT DISTRIBUTION IN GUINEA-PIG LIVERS OF CHEMICALLY KNOWN FAT CONTENT

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ONE TEXT FIGURE AND ONE PLATE (FOUR FIGURES)

Systematic observations upon the distribution of sudanophile fat in the livers of normal, well-nourished guinea-pigs are not available in the literature. Under the circumstances, advantage has been taken of the opportunity to carry out such observations upon a group of male guinea-pigs in which the fat (total lipid) content of the liver has been determined chemically. The chemical and other data upon these animals are recorded in detail elsewhere (Marble, Grafflin and Smith, '40), and the numerical designations there given to the animals have been retained in the present study. Differential analyses of guinea-pig liver fat show that only a small fraction of the total lipid is present as neutral fat. It is the distribution of this small neutral fat fraction which is the subject of the present study.

MATERIAL AND METHODS

For a period of three weeks before sacrifice, the guinea-pigs were maintained in the laboratory upon a diet of Purina Rabbit and Dog Chows, fresh carrots and lettuce; all elements of the diet were available in excess at all times. Guinea-pigs nos. 1 to 12, which were approximately 6 months old, weighed from 620 to 980 gms., average 860 gms. They were sacrificed on June 13 and 15, and the histological observations were completed within the following 2 weeks. Guinea-pigs nos. 25, 26 and 27, which were 3 months old, weighed 440, 425 and 400

gms. respectively. They were sacrificed on November 7, and the observations were completed within the following month. The tissues (transections of entire liver lobes) were fixed in 10% neutral formalin. Frozen sections were cut at 10 μ and stained with a saturated solution of Sudan III in 70% alcohol containing 1% KOH. The sections were mounted in glycerine and studied at low power ($\times 120$) and with oil immersion ($\times 900$). Preparations from the same block of liver tissue proved to be essentially equivalent with respect to the amount and distribution of fat, although slight variations were noted. The fluorescence microscope employed for certain of the observations has been described elsewhere (Grafflin, '39). The fat content given for each animal below represents the total lipid content of the liver.

OBSERVATIONS

Livers of guinea-pigs 6 months old

Study of unstained frozen sections. 1. With the ordinary microscope. Study of unstained frozen sections shows that in all animals the hepatic cells, Kupffer cells and bile duct epithelium are uniformly devoid of recognizable pigment. The readily identifiable fat droplets, irrespective of size, are colorless. 2. With the polarizing microscope. The hepatic parenchyma of all animals reveals an almost complete absence of birefringent material. Small birefringent granules, few in number, are found scattered here and there in the liver substance. The granules usually occur singly, but occasionally form small aggregates; these granules and aggregates are not located with regularity in any particular region of the lobules. The number of birefringent granules varies somewhat from liver to liver, and in different frozen sections from the same liver. 3. With the fluorescence microscope. The principal point of interest for the present problem is the fact that the fat droplets, irrespective of size, are invariably non-fluorescent, which is in keeping with the general experience of other investigators with neutral fat droplets in various tissues (Haitinger, '34; Hamperl, '34).

Study of frozen sections stained with Sudan III. Guinea-pig no. 1 (fat content 1.94%). There is only a moderate amount of histologically demonstrable fat in this liver, and an approximate idea of its amount can be obtained from figure 2. The lobules may contain somewhat, or even considerably, less fat than is shown in the figure; the presence of slightly greater amounts is only rarely observed. There is a tendency for the fat droplets to be confined largely to the central halves of the lobules; occasionally it is located primarily in the intermediate zone, but never in the peripheral zone, which—with the exception of an occasional small droplet—is devoid of fat. Study with oil immersion reveals, in many of the hepatic cells, a variable number of quite small fat droplets, much smaller than those seen in the figure. Occasional hepatic cells contain a large amount of fat—numerous droplets, highly variable in size, and sometimes quite large. Two such heavily laden cells may occur side by side. The immediately adjacent hepatic cells usually contain little or no fat, and it must be emphasized that the majority of the hepatic cells are fat-free. In cells containing only a small or moderate amount of fat, usually in fine droplets, these droplets are commonly localized predominantly or exclusively in the basal portion of the cell, i.e., in close proximity to the adjacent sinusoidal wall. An apical localization is never encountered. Not infrequently the Kupffer cells contain a variable number of fat droplets, and in a few instances are rather heavily laden with them. However, the large majority of these cells are fat-free. Many of the larger fat droplets in the hepatic cells appear to have been formed by the coalescence, with incomplete fusion, of numerous smaller droplets.

Guinea-pig no. 2 (fat content 2.62%). Here again, there is only a moderate amount of fat in this liver. It is commonly confined for the most part to the central half of the lobules, with only an irregular scattering of droplets in the more peripheral zone. Rarely does the amount of fat exceed that shown in figures 3 and 4. It may, on the other hand, be somewhat or very much less in amount, and in such instances may

be confined to the immediate vicinity of the central vein. Hepatic cells heavily laden with fat (fig. 4) are found here and there in the liver substance; they are usually in the central or intermediate thirds of the lobule, but may be more peripheral in location. As in guinea-pig no. 1, oil immersion reveals numerous fat droplets much smaller than those shown in the figures; they are commonly localized in the basal zone of the hepatic cells (next to the sinusoidal wall but definitely within the hepatic cells, not in a hypothetical perivascular lymph space, of which there is here no evidence). Kupffer cells containing fat in variable amounts are occasionally observed, but the great majority of these cells are fat-free, as are the majority of the hepatic cells.

Guinea-pig no. 3 (fat content 2.40%). This liver contains very little fat indeed, and what little there is tends to be localized for the most part in the hepatic cells in the vicinity of the central veins. Hepatic cells heavily laden with fat are only rarely encountered, and are never peripheral in position. A basal localization of very small fat droplets in the hepatic cells is not infrequently noted. Kupffer cells containing fat are few in number. It is to be emphasized that some lobules are essentially devoid of sudanophile fat.

Guinea-pig no. 4 (fat content 2.52%). As in guinea-pig no. 3.

Guinea-pig no. 5 (fat content 2.49%). This liver contains considerably less fat than nos. 1 and 2, but definitely more than nos. 3 and 4. Except for its larger amount, the fat is distributed essentially as in no. 3, and here again some of the lobules are practically devoid of it. In occasional instances, the fat around the central vein is roughly equivalent in amount to that illustrated in figure 5.

Guinea-pig no. 6 (fat content 3.03%). The total amount of fat in this liver is approximately the same as that in guinea-pig no. 5. The amount of fat in different lobules is quite variable, and some lobules are almost devoid of it. In general the fat is localized primarily in the central zone. However, rather well-delimited areas of fat deposition are occasionally encountered in the intermediate zone; and, in two instances

noted, the fat is confined almost exclusively to the peripheral zone, with only scattered droplets in the intermediate zone and essentially no fat in the central zone. The maximum fat deposition observed in the preparations is illustrated in figure 5. Isolated hepatic cells heavily laden with fat are very rare indeed. Occasional Kupffer cells contain small fat droplets, variable in number and at times rather abundant.

Guinea-pig no. 7 (fat content 2.35%). The amount of fat in this liver is very small (less than in nos. 5 and 6, about the same as in nos. 3 and 4), and the distribution is essentially the same as in guinea-pig no. 3. Fat-containing Kupffer cells are often confined to the region of fat deposition around the central vein. They may occur, however, in the immediately peripheral zone (adjacent to the portal areas), the hepatic cells being fat-free.

Guinea-pig no. 8 (fat content 2.66%). The total amount of fat in this liver is approximately the same as in nos. 3, 4 and 7. Fat-laden hepatic cells are few in number. Appreciable accumulations of fat around the central veins are not observed. Occasionally rather well-delimited areas of fat deposition (suggestive of fig. 5) are encountered in the intermediate or peripheral zones. The great majority of the hepatic and Kupffer cells are fat-free. A predominant or exclusive localization of small fat droplets in the basal region of the hepatic cells is not infrequently observed. Hepatic cells heavily laden with fat may be either mononucleate or binucleate; they may occur singly or in pairs. In these cells it is not unusual to find a fat droplet with a diameter equal to, or even considerably larger than, that of the nucleus; at times the nucleus is markedly eccentric in position.

Guinea-pig no. 9 (fat content 2.79%). The amount of fat in this liver is roughly equivalent to that in nos. 5 and 6. Some lobules are essentially devoid of fat; in others one finds a scattering of hepatic cells heavily laden with fat. These cells may occur singly or in small groups; when present in a lobule in appreciable numbers they are usually located in the

central or intermediate zones. Such cells are predominantly mononucleate; for example, out of fifty consecutive heavily laden hepatic cells which were studied, forty-seven proved to be mononucleate, and only three binucleate. No cells containing more than two nuclei are encountered. The predominant or exclusive location of varying numbers of small and/or medium-sized fat droplets in the basal zone of the hepatic cells is not infrequently observed. However, the great majority of the hepatic and Kupffer cells are fat-free.

Guinea-pig no. 10 (fat content 3.10%). This liver contains very little fat, and is comparable with nos. 3, 4, 7 and 8. No significant fat deposits are observed in the central, intermediate or peripheral zones. Fat-laden hepatic cells are few in number. Some of the hepatic cells (more of these than of the heavily laden cells) contain one or more small or medium-sized droplets, confined for the most part to the basal region. These cells are distributed more or less at random in the lobules. Most of the hepatic and Kupffer cells are fat-free.

Guinea-pig no. 11 (fat content 2.80%). Essentially the same as guinea-pig no. 10. Appreciable fat deposits in the central zone are very infrequent. As stated above (no. 9), hepatic cells heavily laden with fat are predominantly mononucleate. As a supplement to this observation, a particular study of binucleate cells in the present animal demonstrated that they are for the most part fat-free. In this liver Kupffer cells containing a few small fat droplets are somewhat more numerous than usual.

Guinea-pig no. 12 (fat content 2.86%). The amount of fat in this liver is equivalent to that in nos. 3, 4, 7, 8, 10 and 11. The number and distribution of fat-laden hepatic cells is the same as in no. 10. No zonular fat deposits are observed. Occasionally a fair number of the hepatic cells in a rather well delimited area contain moderate amounts of fat; these areas include only a small portion of the lobules in question, and are not arranged concentrically around the central vein. The great majority of the hepatic and Kupffer cells are fat-free.

Livers of guinea-pigs 3 months old

Guinea-pig no. 25 (fat content 2.57%). 1. The great bulk of the hepatic cells are entirely devoid of fat. Under oil immersion cells are infrequently encountered which contain one to several droplets, usually very small. The position of these droplets in the cell is so variable that one cannot speak of a characteristic location. Also, these cells may be found anywhere in the lobule, and usually occur singly. (2) A zonular deposition of fat in the lobules is never observed. (3) Isolated hepatic cells containing moderate or large amounts of fat are of quite infrequent occurrence; they may occur anywhere in the lobule. (4) A considerable number, but still a minority, of the Kupffer cells contain fat. The droplets are usually rather small, and vary from one or two up to quite large numbers in a given cell. The hepatic cells adjacent to these fat-containing Kupffer cells are almost invariably devoid of fat. Furthermore, hepatic cells may contain small, intermediate or large amounts of fat without any being present in the lining cells of the adjacent sinusoids. (5) Pigment is nowhere observed in the liver except in a rare Kupffer cell which, in addition to fat droplets, contains a number of small black pigment granules.

Guinea-pig no. 26 (fat content 2.43%). (1), (2), (3), (4) and (5) as in guinea-pig no. 25, except that fat-laden hepatic cells are more numerous, and the number of fat-containing Kupffer cells is definitely somewhat less and they contain on the whole less fat. (6) At times a moderate amount of fat is found in the connective tissue of the portal areas, and in such instances many of the Kupffer cells in the peripheral portions of the immediately adjacent lobules may show unusual amounts of fat, and a number (minority) of the hepatic cells may likewise contain fat, though in small amounts.

Guinea-pig no. 27 (fat content 2.85%). (1), (2), (3) and (4) as in guinea-pig no. 25, except that the fat-containing Kupffer cells are less numerous and contain less fat; (6) as in guinea-pig no. 26. No pigment is observed anywhere in the liver.

The distribution of fat-containing Kupffer cells in relation to fat-containing and fat-free hepatic cells

As described above, a relatively small number of Kupffer cells in all of the animals contain fat in variable amounts. These fat-containing cells are frequently found immediately adjacent to hepatic cells containing small, moderate or large amounts of fat. On the other hand, they are very often observed, either singly or in small groups, in areas where the hepatic cells are entirely fat-free. The reverse situation is equally common: hepatic cells may exhibit the whole range of fat content without any fat, even in the smallest amounts, being detectable in the cells lining the adjacent sinusoids. Particular attention has been directed to this aspect of the problem, and the abundant observations necessitate the conclusion that, in the liver of the guinea-pig, there is no essential relationship between fat deposition in the Kupffer cells on the one hand, and in the hepatic cells on the other.

Fat droplets in the bile duct epithelium

Guinea-pigs nos. 1 to 12. Not infrequently one observes small, well-stained fat droplets in the cells lining the bile ducts. These droplets vary somewhat in size and considerably in number; in a given duct they may be entirely absent, or may be present in a few, many or all of the cells. They are predominantly basal or paranuclear, rarely apical, in position.

Guinea-pig no. 25. The epithelial cells of the bile ducts contain fat droplets in only a minority of instances. The droplets are few in number, are very small, and are usually paranuclear or apical in position. Guinea-pig no. 26. Some cells of the bile ducts contain one, two or several small fat droplets, which may be apical, paranuclear or basal in position. In one very large duct many of the cells contain a small cluster of minute droplets, always located immediately above the nucleus, with no fat elsewhere in the cytoplasm. Guinea-pig no. 27. In the smaller bile ducts a variable number of the cells contain from one or several to numerous small fat droplets,

chiefly basal and/or paranuclear in position. The cells of the larger bile ducts seldom contain fat (at most several small paranuclear droplets). In several canals of Hering the cells just distal to the connection with the hepatic cell cords are observed to contain unusually large amounts of fat (numerous small and medium-sized droplets, chiefly basal and paranuclear in position). As one proceeds along the canal, in the direction of the larger bile duct, the fat usually diminishes more or less rapidly in amount, but may persist in quantity for a considerable distance.

Intranuclear fat droplets

Guinea-pigs nos. 1 to 12. In occasional instances well stained sudanophile fat droplets are observed within the nuclei of the hepatic cells. Guinea-pig no. 1: very occasionally the nuclei contain one or more (never many) fat droplets; these are usually minute, but may be of fair size. No. 2: two instances noted (1—one small droplet; 2—one small, one fairly large droplet). No. 3: two instances noted; cells otherwise contain no fat. No. 4: one instance, with one small droplet; no other fat in cell. No. 5: one instance, with eight minute droplets and one of fairly good size; the cell contains in addition rather numerous small droplets, confined to the basal region. No. 7: one instance, with one small droplet; no other fat in cell. No. 8: one instance, with one rather large droplet; no other fat in cell. In the remaining five animals no intranuclear fat droplets are observed.

Guinea-pigs nos. 25, 26 and 27. For each of these animals a single frozen section (cross-section of an entire lobe of the liver, from the same lobe in each case) was carefully searched for intranuclear fat droplets. No. 25: The nuclei contain fat in twenty-two instances; eight of these nuclei are illustrated in figure 1, and the remaining fourteen exhibit essentially the same appearance as one or the other of this group. These intranuclear fat droplets are the only sudanophile fat visible in the cells with three exceptions (one to three droplets in cytoplasm). Two of the fat-containing nuclei are in the same

binucleate cell. No. 26: Five of the nuclei contain fat. The cytoplasm of the cells is almost or entirely devoid of fat. No. 27: The nuclei contain fat droplets in nine instances. The cytoplasm of these cells is fat-free.

The fat droplets here described are always free in the nucleoplasm (i.e., are never intra-nucleolar). Also, it should be emphasized that the fat-containing nuclei are regularly located in hepatic cells which contain little or no sudanophile fat in the cytoplasm. Fat droplets are never observed in the nuclei of the Kupffer cells or of the bile duct epithelium.

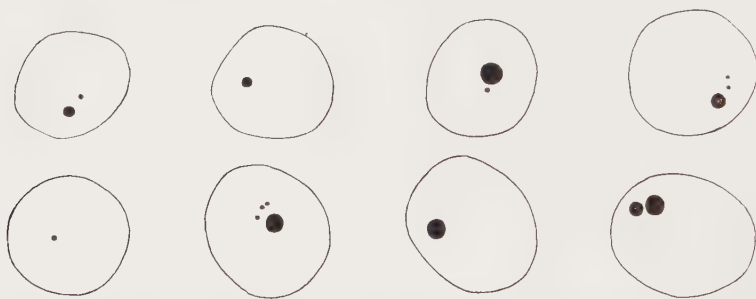


Fig. 1 Intranuclear fat droplets in hepatic cell nuclei of guinea-pig no. 25. Frozen section, stained with Sudan III. Camera lucida drawings at $\times 2375$; as reproduced, $\times 1900$.

The occurrence of fat droplets in the nuclei of hepatic cells has been reported previously for man, dog and rat. A full discussion of the problem, with references to the pertinent literature, can be found in the recent article by Berg ('35). Chemical analyses of the lipids in nuclei of muscle, tumor and pus cells (but not of liver) have recently been reported by Stoneburg ('39), who found the nuclei to be especially rich in phospholipids and cholesterol.

Histologically demonstrable fat in relation to known chemical fat content of liver

It is quite evident from the present study that the relative amounts of histologically demonstrable (sudanophile) fat in different livers are not an accurate index of the relative

total lipid contents as determined by chemical methods. For example, guinea-pigs nos. 1 to 12 fall into three groups on the basis of the amount of histologically demonstrable fat, although the distribution of this fat may vary. In each group the livers are equivalent to such an extent that any attempt to distinguish between them would be unjustified. Group 1, exhibiting the largest amount of fat, comprises nos. 1 (1.94%) and 2 (2.62%). Group 2, containing considerably less fat than group 1 but definitely more than group 3, comprises nos. 5 (2.49%), 6 (3.03%) and 9 (2.79%). Group 3 comprises the remaining seven animals, with fat contents varying from 2.35 to 3.10%. No. 26 (2.43%) exhibits a larger amount of sudanophile fat than no. 25 (2.57%), and no. 27 (2.85%) a larger amount than no. 26.

SUMMARY

Systematic histological observations have been made upon the distribution of sudanophile fat in the livers of fifteen normal, well-nourished guinea-pigs in which the fat (total lipid) content of the liver has been determined chemically. The majority of the hepatic cells are devoid of fat. When a zonular distribution of fat is observed in the lobule, it is almost invariably located in the region surrounding the central vein. Hepatic cells heavily laden with fat are regularly present; they are few in number, and their distribution is quite irregular. A relatively small number of Kupffer cells in all of the animals contain fat in variable amounts. However, there is no essential relationship between fat deposition in the Kupffer cells on the one hand, and in the hepatic cells on the other. The epithelial cells of the bile ducts sometimes contain fat droplets, usually in small numbers.

Well-stained sudanophile fat droplets are occasionally observed in the nuclei of the hepatic cells (in ten out of fifteen animals).

It is quite evident from the present study that the relative amounts of histologically demonstrable (sudanophile) fat in different livers are not an accurate index of the relative total lipid contents as determined by chemical methods.

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PLATE 1

EXPLANATION OF FIGURES

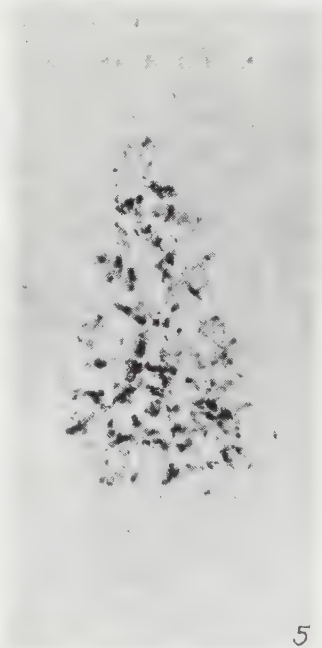
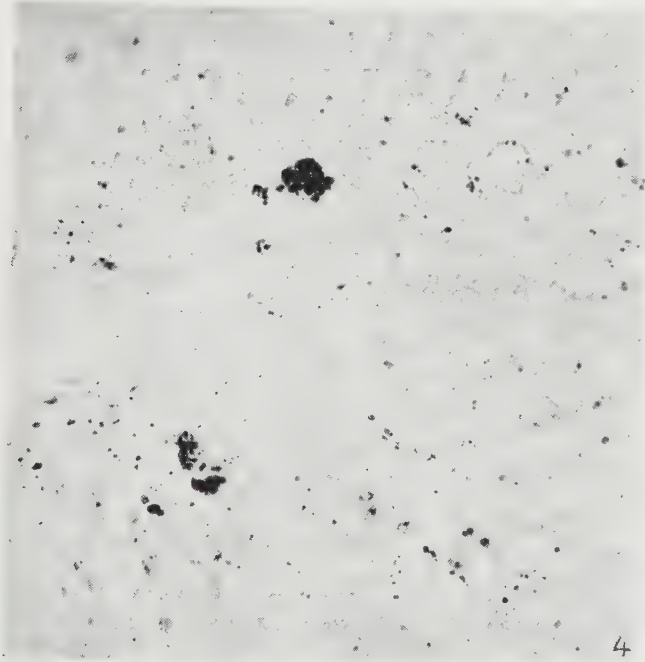
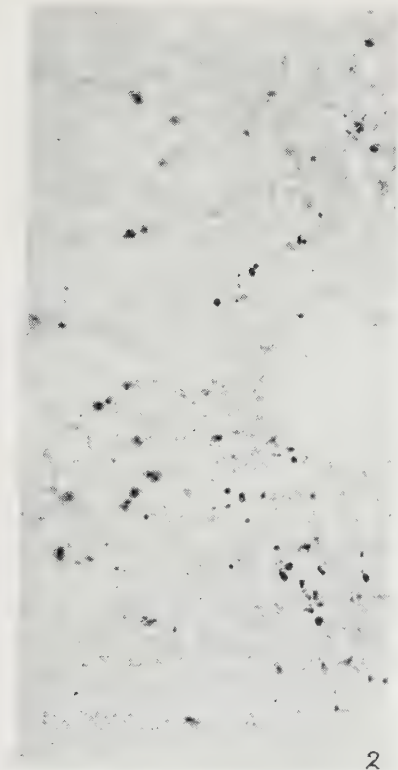
2 Guinea-pig no. 1. Fat deposition in the central zone of the lobule. The large central vein is seen in cross-section to the right of the figure.

3 Guinea-pig no. 2. Fat deposition confined for the most part to the central half of the lobule. The small central vein (not apparent in the photograph) is located to the left and below the center of the figure.

4 Guinea-pig no. 2. Fat deposition confined for the most part to the central half of the lobule. The branched central vein, cut lengthwise, is seen to the left of the figure. The concentration of fat droplets above center represents three hepatic cells heavily laden with fat; below center are seen two such fat-laden cells. The lobule is immediately peripheral (subcapsular) in position (the position of the capsule is beyond the right edge of the figure).

5 Guinea pig no. 6. Localized area of fat deposition in the central zone of the lobule.

All photographs made from frozen sections (10μ) of formalin-fixed material; stained with Sudan III (no counterstain); $\times 140$.



A FURTHER MORPHOLOGICAL AND BIOCHEMICAL STUDY ON THE INTRANUCLEAR CRYSTALS IN THE HEPATIC CELLS OF THE DOG—THE PURE BREED AND HYBRID DALMATIAN

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TWO PLATES (SIXTEEN FIGURES)

In an earlier paper (Weatherford, '38) on the intranuclear crystals in the hepatic cells of dogs, it was reported that four pure breed Dalmatian dogs had fewer nuclei with crystals than other breeds (Dalmatians, average 0.26% of nuclei containing crystals; other breeds 1.27%). This occurrence in the liver of the Dalmatian dog of a diminished number of nuclei with crystals seemed too consistent to be the result of a mere chance condition. Because of the unique capacity of this breed to excrete uric acid, and the evident purine origin of the intranuclear crystals, it seemed desirable to determine whether or not in a larger series of Dalmatians there would be a similar consistency of few nuclei containing crystals. Fortunately we have had access to experimental material from two different kennels, the stocks of which were totally unrelated, as well as detailed information about the ages and ancestry of the animals. In addition, pieces of liver were obtained from several Dalmatian-Collie hybrids which differed markedly with respect to their uric acid excretion. Biochemical data upon most of the animals studied have been collected and correlated with the frequency of the occurrence of hepatic intranuclear crystals.

From their comparative studies on purine metabolism, A. Hunter and his collaborators ('14 and '20) showed that in man

and the chimpanzee most of the purine derivatives were excreted as uric acid, while in the various other mammals examined there was a further degradation to allantoin. They derived from their extensive data what they termed the "uricolytic index",¹ an expression of the ratio of the allantoin nitrogen to the sum of the uric acid nitrogen plus the allantoin nitrogen present in the urine of each species. The "index" for the dog was given as 98: for man, 2: and for the chimpanzee, 0.

S. R. Benedict ('16) discovered that the Dalmatian dog was exceptional in excreting a large quantity of uric acid in its urine. He stated that this dog on a purine-free diet eliminated almost as much uric acid per day as would an average-sized man on a similar diet, although the dog weighed only about 10 kg. Uric acid injected subcutaneously was eliminated unchanged in the urine. In the ordinary dog the greater part of uric acid parenterally introduced or formed in metabolism disappears and there is an increase in the allantoin excreted (Spiegelberg, 1898; Swain, '01; Burian and Schur, '01; and others).

It has been thought generally that the presence of an enzyme (uricase) in the liver, spleen, and perhaps other organs, was necessary for the oxidation of uric acid to allantoin. An absence of this uricolytic enzyme has been given as an explanation for the "high" uric acid excretion in man and the chimpanzee and from analogy it might be expected that the same condition held true for the Dalmatian dog. Wells ('18) detected the presence of uricase in the liver of one Dalmatian, indicating that in this animal the "high" excretion of uric acid could not be accounted for by the absence of the enzyme. Klemperer, Trimble and Hastings ('38) made quantitative observations upon the uricase content in the livers of the Dalmatian and other breeds of dogs and found it to be substantially the same in both groups. Bollman, Mann and Magath ('25) found that the destruction of uric acid in the non-

¹ Uricolytic index = $\frac{\text{allantoin N}}{\text{uric acid N} + \text{allantoin N}} \times 100$.

Dalmatian dog depended upon the liver, since a complete extirpation of this organ resulted in increased excretion of uric acid.

The inheritance of "high" uric acid excretion by hybrid Dalmatians has been studied by Onslow ('23) and Trimble and Keeler ('38). From their experiments, involving a large number of dogs, Trimble and Keeler concluded that "high" uric acid excretion was "inherited as an almost completely recessive, non-sex-linked unit-character, dependent for its expression upon the presence of a single pair of Mendelizing genes."

In the present report will be offered the results of observations on the morphology and systematic counts of nuclei in the hepatic cells, with a determination of the percentage containing intranuclear crystals. Evidence will be set forth also, showing a parallel between counts of nuclei with crystals and the uric acid excretion of the animals.

MATERIAL AND METHODS

Seventeen dogs (thirteen pure breed Dalmatians from two different kennels, three third generation Dalmatian-Collie hybrids, and the male Collie ancestor of the hybrids) were used. The ages of the dogs ranged from puppies 1 day old to animals 13½ years old. Pieces of liver were excised either at operation or immediately after the death of the dog from a lethal dose of Nembutal. Tissue from all animals was fixed in a 10% aqueous solution of formaldehyde and from some dogs additional pieces of liver were fixed in Heidenhain's "susa" mixture. Embedding was in paraffin. Sections were cut 5 μ thick, mounted serially and stained with hematoxylin and eosin or by the Heidenhain "azan" method. Hepatic cell nuclei were counted in successive microscopic fields, after the manner employed for making differential counts of blood cells. A total of 10,000 nuclei were counted for every dog—1000 in each of ten sections, spaced far enough apart so as not to include the same nuclei. The counts were made independent of the chemical measurements.

In the laboratory the adult animals on Benedict's standardized purine-free diet were confined to metabolism cages for several days and the urine collected over 24-hour periods. Younger animals receiving mother's or cow's milk were put into collecting cages for several periods of 12 hours each. The registered Dalmatians were anesthetized at their kennel with Nembutal given intravenously, and the urine from the bladder was withdrawn into a large syringe. It was transferred to bottles, covered with toluene, and transported to the laboratory where chemical measurements were made within a few hours. These registered animals had been receiving a general mixed diet, but were without food for 18 to 24 hours prior to the time the urine was collected.

Uric acid and creatinine in urine was determined by the methods of Folin and Trimble ('24) and Folin ('25). Blood uric acid (using laked filtrates) was measured by the Folin ('33) method. The procedure of Larson ('32) was employed for the measurement of allantoin.

OBSERVATIONS

1. *The nucleus of the hepatic cell.* The average nucleus of the hepatic cell of the Dalmatian dog measures between 7 and 8 μ in diameter and each contains one or more acidophilic nucleoli. In counts of 4000 nuclei, 1000 in sections of liver from each of four dogs ranging in age from 1 day to 10 years and 3 months old, 3658 or 91.45% contain but 1 nucleolus; 283—2; 52—3; and only 7 have 4 nucleoli. No deduction is made as to relationship between age and number of nucleoli, because of the unappreciable differences in the individual counts. These true nucleoli vary in diameter from 1 to 2.5 μ and generally the largest are seen in nuclei containing a single nucleolus.

The nuclear reticulum is delicate and much of the scant chromatic substance is condensed in block-like masses on the inner surface of the nuclear membrane. Sometimes chromatin granules are clustered about a nucleolus giving the deceptive appearance of a thickened envelope. Obviously all these struc-

tures are compressed peripherally when a nucleus contains a crystal. The nuclear membrane is thin, faintly basophilic and very elastic. While most of the nuclei are round in section, others, because of shrinkage, become wrinkled or corrugated along with their enclosing membrane. When the length of a crystal exceeds the diameter of a nucleus, the membrane becomes distorted and sometimes even elliptical in shape. The great elasticity of the nuclear membrane is really appreciated when the average size nucleus is compared with one containing an exceptionally long crystal.

2. *The occurrence and morphology of the crystals.* Surrounded by clear areas, crystals are found within vacuoles in a small percentage of nuclei (fig. 1). The interfacial membrane separating these vacuoles from the surrounding nucleoplasm varies in thickness with the size of the crystal it encloses. When the vacuole is of small size enclosing a tiny crystal, the thicker interfacial membrane shows a slight basophilia—staining light bluish with hematoxylin. As the crystal enlarges, the membrane becomes thinner, until the one surrounding the largest vacuole may be imperceptible. Aside from the crystals, the vacuoles are usually devoid of other stainable substance.

While the majority of intranuclear crystals occur singly (figs. 1 to 4), other nuclei enclose several (figs. 7 and 8). These multiple crystals are observed both in the same and in separate vacuoles and sometimes they are so large that those adjacent become contiguous to one another and the separating membrane very distinct. In other nuclei the separating membranes break down and disappear. The question arises now whether multiple crystals are formed in situ, namely, in the same vacuole or through fusion. Morphological appearances are seen which might be interpreted in either way, but being unable to study them in fresh tissues opinion is reserved.

Crystals are found mostly in normal resting nuclei although occasionally one appears in a dividing nucleus or in one undergoing degeneration. They are seldom seen in binucleate cells and almost never in both the nuclei.

The presence of intranuclear crystals does not seem to affect the cytoplasm although occasionally a crystal is observed partially extruded from a nucleus. Whether this extrusion represents a real condition or an artifact produced by technique cannot be stated. Only in a few instances are free crystals detected in the cytoplasm—where they usually exhibit signs of dissolution with broken angles and eroded or serrated edges.

Although crystals generally show a regular form, in the sections of liver from one animal having almost twice as many nuclei containing crystals as the other Dalmatians, an appreciable number are irregular. The kennel-keeper reports that this particular dog (no. 12) was subject to what he terms periodic “fits” and it is possible that this condition affects its metabolism. Twinning of crystals occurs not infrequently; triplets are observed sometimes in cross-section, and occasionally we see bizarre forms and crystalline masses (figs. 15 and 16).

As in the livers of other breeds of dogs the intranuclear crystals in the Dalmatian show gradations in size from the minute to those several times as long as the diameter of most nuclei. The average length is between 7 and 12 μ —the longest measures 32—and the widths seldom exceed 5 μ .

Crystals appear as hexagonal prisms (figs. 9 and 10) but through cleavage the regular cross section may be modified (fig. 11). When very long, they sometimes become slightly curved (fig. 4), probably caused by the spatial relations in a nucleus. Some show transverse fracture, giving a staircase appearance (fig. 6).

3. *Numbers of crystals.* From counts of nuclei in sections of liver from thirteen pure breed Dalmatian dogs—1 day to 13½ years old—intranuclear crystals are found in twelve, none appearing in the nuclei of a puppy 1 day old. In the individual dogs the number of nuclei containing crystals deviate—from 5 to 67 in 10,000 nuclei. The lowest count of 5 is obtained in

a day old puppy, a litter-mate to the one in which no crystals are found and the highest count appears in a dog 10 years and 3 months old. The count in the latter, while nearly twice as great as obtained for any other pure breed Dalmatian, is only about one-half the average for non-Dalmatian dogs (Weatherford, '38).

TABLE 1

A tabulation of the age, sex, and the number of hepatic cell nuclei in counts of 10,000 containing crystals

NUMBER OF ANIMAL	AGE	SEX	NUMBER OF NUCLEI IN 10,000 WITH CRYSTALS	REMARKS
2	1 yr., 5 mos.	M	39	
4	4+ yrs.	M	37	
13	9 yrs., 7 mos.	M	27	Son of no. 11.
12	10 yrs., 3 mos.	M	67	Periodic "fits."
11	10 yrs., 10 mos.	M	17	Son of no. 10.
10	13 yrs., 6 mos.	M	22	
8	1 day	F	0	} Litter mates
7	1 day	F	5	
15	1 yr., 6 mos.	F	22	Daughter of no. 16.
1	3 yrs., 3 mos.	F	14	
3	3 yrs., 11 mos.	F	19	
14	7 yrs., 4 mos.	F	27	Daughter of no. 10 and no. 16.
16	9 yrs., 8 mos.	F	35	Same grandfather as no. 12.
6	14 wks.	M	28	Dalmatian-Collie F ₃ hybrid.
9	10 wks.	F	29	Dalmatian-Collie F ₃ hybrid.
5	9 wks.	F	66	Litter mate of no. 6.
17	13+ yrs.	M	70	Pure breed Collie. Great grandfather of hybrids 5, 6 and 9.

From the animals examined, neither age nor sex seem to be factors in determining the number of nuclei containing crystals. For instance, in one female dog (1 year and 6 months old) is counted the same number of nuclei with crystals as in a male (13 years and 6 months old)—both having 22 in 10,000. And conversely, a young male has approximately as many crystals as a female nearly twice his age.

Three male dogs representing three generations show a nearness in count.

Grandfather,	No. 10,	13 years and 6 months	22 in 10,000;
Father,	No. 11,	10 years and 10 months	17 in 10,000;
Son,	No. 13,	9 years and 7 months	27 in 10,000.

Singularly enough two dogs—no. 12 and no. 16, having a common grandfather, show a count of nuclei with crystals above the average. Also, of some interest is the showing in one female dog, no. 14—the daughter of no. 10 ♂ low in count of nuclei with crystals and of no. 16 ♀ above the average in count, who seems apparently to follow the low count of the father.

All pure breed Dalmatians have a “high” daily output of uric acid (see table 2). Among the F_3 generation Dalmatian-Collie hybrids, in dog no. 5 with a “low” daily excretion of uric acid, 66 nuclei in 10,000 are counted with crystals, while in dog no. 6, a litter mate, and eliminating more than five times as much uric acid per day, only 28 nuclei in 10,000 contain crystals. A 10-week-old F_3 hybrid (no. 9) having the same male Collie ancestor as dogs nos. 5 and 6 and with a “high” uric acid output, gives a count of 29 nuclei containing crystals. The Collie ancestor of these three hybrids (13+ years old) over a period of years consistently shows a “low” daily excretion of uric acid. In the sections of liver from this Collie dog 70 nuclei in 10,000 contain crystals—a count greater than obtained for any single Dalmatian and nearly three times the average for this breed.

4. *Biochemical.* The quantity of uric acid excreted by a dog in 24 hours is divided by the weight of the animal and recorded in the third column of table 2 in milligrams per kilogram per day. It will be noticed that the data for the pure breed Collie (no. 17) and the “low” uric acid excreting Dalmatian-Collie hybrid (no. 5) are in sharp contrast to those for the other animals studied.

The registered Dalmatians obtained from a distant kennel could not be placed in a metabolism cage; therefore, the urine

was collected from the bladder (at the time of death) and analyzed later for both uric acid and creatinine.² The ratios of the nitrogen content of these two substances is computed and recorded in column 4 of table 2. Unpublished statistics (H. C. Trimble) show that for adult non-Dalmatian dogs this ratio ranges between 0.1 and 0.2, while for Dalmatians it is between 0.5 and 1.1. For puppies and partly grown animals the ratios are numerically larger because the excretion of uric acid is at a somewhat greater rate per day while that of creatinine is at a distinctly lesser rate. Therefore, ratios of 0.5–0.6 have been observed for puppies of non-Dalmatian types while ratios between 3.0 and 4.0 have been found in Dalmatians. Against this background it is evident that the ratio of the urine uric acid nitrogen to the urine creatinine nitrogen of dog 17 is in a different category from those of dogs nos. 1–3 and 10–16. A comparison of the ratio for dog 5 with those for dogs 6–9 shows a similar distinction.

The uric acid content of the blood is determined in a number of instances. Table 2 shows that the value obtained for the Collie dog, no. 17, is distinctly lower than that for any of the pure breed Dalmatians.

That the rate of excretion of allantoin by dogs of both Dalmatian and non-Dalmatian breeds increases with the nitrogen content of a purine-free diet is established (H. C. Trimble) in unpublished experiments. On the other hand the uric acid excretion is influenced relatively little by the nitrogen intake. Therefore, measurement of the rate of allantoin excretion per day is not as helpful as the uric acid data in making biochemical distinctions between the animals studied. Because of this fact and the tediousness of the analytical methods involved only a few data upon allantoin excretion have been collected for the dogs of this series.

5. *Genetic data.* The genetic characteristics of some of the animals used in this investigation have already been described

²It has been demonstrated (A. Hunter, '28) that the excretion of creatinine nitrogen is quite uniform per hour; therefore it can be used as an index for comparing the rates of excretion of other substances.

by Trimble and Keeler ('38). In table 3 are listed the designations used for the animals in this and in the previous report. The identification numbers of the registered Dalmatians are appended to a copy of this report which is deposited in the library of the Harvard Medical School for permanent preservation.

TABLE 2

A comparison of allantoin and uric acid excretions and the uric acid-creatinine ratios with the number of hepatic cells containing intranuclear crystals

NUMBER OF ANIMAL	ALLANTOIN EXCRETION	URIC ACID EXCRETION	RATIO URIC ACID N	BLOOD URIC ACID	NUCLEI WITH CRYSTALS
			CREATININE N		
	<i>mg./kg./day</i>			<i>mg. per cent</i>	<i>per 10,000</i>
17	22	4.6	0.11 ^a	0.3	70
5	—	8 ^b	0.52 ^b	—	66
2	24	35	0.86 ^a	0.76	39
1	28	35	1.09 ^a	0.5	14
3	—	30	0.86 ^a	1.5	19
4	—	—	—	—	37
7	—	38 ^c	3.6 ^c	—	5
8	—	38 ^c	3.6 ^c	—	0
9	—	53 ^d	3.5 ^d	—	29
6	—	42 ^e	3.08 ^e	—	28
15	—	—	0.67 ^f	0.82	22
14	—	—	0.50 ^f	0.75	27
13	—	—	0.64 ^f	0.73	27
16	—	—	0.57 ^f	0.72	35
12	—	—	0.95 ^f	0.62	67
11	—	—	0.97 ^f	0.73	17
10	—	—	0.60 ^f	0.89	22

a = Ratio based on excretion for 24-hour period.

b = At 25 days of age.

c = Average of litter mates at 18 days of age.

d = At 15 days of age.

e = At 20 days of age.

f = Ratio based on urine in bladder at death.

TABLE 3

Designations of experimental animals

NUMBER USED IN THIS PAPER	NUMBER USED BY TRIMBLE AND KEELER ('38)
17	P ₁ ¹
5	F ₂ ⁵
3	P ₁ ²
6	F ₂ ⁴

DISCUSSION

The observations show the occurrence and a similar morphology of the intranuclear crystals in the hepatic cells of the Dalmatian dog as have been described for other breeds. In always having a low count of nuclei containing crystals, the Dalmatian is unique and the figures obtained for this breed are so at variance with those for other dogs as to be significant.

Intranuclear crystals, even those several times the length of the diameter of an average-size nucleus, do not apparently affect the cytoplasm. The cells appear to have adjusted themselves to the enclosed crystals, indicating that the phenomenon is not recent but has likely been evolved over a long period. Additional evidence in favor of their acclimation is the finding of crystals in the hepatic nuclei of other Canidae—wolves, foxes, jackals and non-domestic dogs (Weatherford, '39).

Berg ('34) in discussing the formation of intranuclear crystals assumed that the formative material for them was not present in the normal nucleus but wandered in through the nuclear membrane, concentrated in fluid droplets and subsequently crystallized. It was stated further that intranuclear vacuoles without crystals were found in the livers of forms other than the dog (cold blooded animals and mammals) and he concluded that even though similar metabolic conditions were possible, crystallization did not take place because of either the lack of sufficient concentration or the presence of disturbing substances. While these ideas of Berg's are suggestive, they must be taken as purely speculative and not necessarily satisfying all the conditions for crystal formation.

An attempt to link the crystals with homogeneous, rounded, intranuclear inclusion bodies in the liver and kidney of the dog by Nicolau and Kopciowska ('36) was not supported by very secure evidence. They adduced that the inclusions were probably formed through the influence of a saprophytic virus in the interior of a nucleus which persisted there for a long time and therefore the age of the dog was a factor. The phenomenon of adaptation was stressed; the cells were stated as being tolerant to the presence of these inclusions and the

function of the organ not compromised. Because both the rounded bodies and the "crystalloids" stained similarly, Nicolau and Kopciowska believed that the crystal-like form resulted from a progressive accumulation.

Cowdry and Scott ('30) saw both intranuclear inclusion bodies and crystals in the livers of a number of dogs, but were careful not to confuse the two. Inclusion bodies experimentally attempted in sixty-eight dogs, but produced in only two, were found far more numerous (in one, 80%, and in the other, 20% of the nuclei) than inclusions and crystals together in any normal animal. They stated that these created bodies were definitely associated with hemorrhage and necrosis in the central and middle thirds of the hepatic lobules and were accompanied by a marked swelling of sinusoidal endothelium and increase of phagocytic cells. Furthermore, Cowdry and Scott's account of these experimentally produced inclusions which they described to be like those in virus diseases, was entirely different from the appearance of the circumscribed bodies seen in the nuclei of presumably normal dogs by Brandts ('09), Berg ('29 and '34) and Weatherford ('38).

It is probable that the inclusion bodies described by Nicolau and Kopciowska, as resulting from virus infection, are the same as those seen in normal dogs. Vacuoles containing condensed staining material and crystallizing masses are encountered not infrequently in the nuclei of many dogs; that these masses represent crystals in formation appears probable and Berg ('34) has inferred the same. Moreover, since intranuclear crystals are observed in dogs of all ages (from 1 day on), it seems reasonable that those present in the youngest could hardly result from a virus infection. Also, if the cause is infectious, there would be visible cytoplasmic injury to the cells with either inclusion bodies or crystals.

Age of the animal and the presence of intranuclear crystals has been emphasized by several writers—Grandis (1889), Brandts ('09), and Nicolau and Kopciowska ('36) who failed to find them in young dogs. In the present study crystals are observed in very young pure breed and hybrid dogs—1 day,

9 weeks, et cetera, and there is no reason to believe they do not exist in puppies of other breeds. The papers of the above authors give no indication that either extensive or systematic counts of nuclei were made and so it is more than likely that if crystals were few in number they were overlooked.

Among earlier investigators—Browicz (1897 to 1899), Herring ('06), Herring and Simpson ('06), Brandts ('09) and Fiessinger ('11) have considered the intranuclear crystals in the hepatic cells of the dog as hemoglobin or a derivative of it, basing their conclusions more on a similarity in staining between the crystals and red blood corpuscles than on any actual effort to identify their real nature. Hemoglobin crystals differ greatly from those in the nuclei of hepatic cells in both form and solubilities. Also, the absence of iron in intranuclear crystals further differentiates them from hemoglobin. Since hemoglobin crystallizes easily in all breeds of dogs, why are there fewer intranuclear crystals in the hepatic cells of the Dalmatian if they are hemoglobinous?

Grandis (1889) considered the intranuclear crystals as derived from the purines of the nucleus and Berg ('29) from more detailed studies concluded that the material for the crystals had passed in through the nuclear membrane and that they were probably largely allantoin, the principal end-product of purine metabolism in the dog. Berg also stated that the crystals belonged in the hexagonal system. The crystallography of allantoin is well known: the International Critical Tables and various handbooks place it in the monoclinic system, which in itself should eliminate allantoin as a possibility. Weatherford ('38) believed that the intranuclear crystals were of purine origin, although the exact product they represent was undetermined.

Considering the consistency in low counts of nuclei containing crystals in the Dalmatian dog, it is believed that the results obtained cannot be adventitious. Our data reveal that the frequency of the occurrence of intranuclear crystals in the hepatic cells of animals of this series varies *inversely* with the rate of excretion of uric acid (table 2). On the other hand

their frequency shows no correlation with the rates of excretion of allantoin in those instances in which this quantity was measured. It has been mentioned earlier that the excretion of allantoin in dogs is greatly varied by changing the protein content of a purine-free diet, while the excretion of uric acid is not influenced. In fact the rate of excretion of uric acid is so definite that it might be characterized as a biochemical constant. These findings indicate quite definitely that intranuclear hepatic crystals are related to uric acid excretion and not to that of allantoin.

SUMMARY

1. The morphology of the hepatic cell nucleus is described for the Dalmatian dog—the average measures between 7 and 8 μ in diameter and contains one to four nucleoli varying in diameter from 1 to 2.5 μ .

2. Intranuclear crystals within vacuoles are found in a small percentage of nuclei and their presence does not obviously affect the cytoplasm.

3. The crystals vary in size from the minute to those several times the length of the diameter of the average nucleus.

4. In the livers of the thirteen pure breed Dalmatians, 331 nuclei containing crystals are counted in a total of 130,000, the deviation in the individual animals being from 5 to 67 in 10,000—the average is 0.2546%. (Average for non-Dalmatians, Weatherford, '38, is 1.27%.)

5. Among F_3 generation Dalmatian-Collie hybrids, dogs having a "high" daily uric acid output show fewer nuclei with crystals than those having a "low" daily excretion.

6. Evidence that the occurrence of intranuclear crystals is related to uric acid excretion and not to that of allantoin is presented.

We acknowledge our indebtedness to the late Ellen de F. T. Gladding of Providence, Rhode Island, and the executors of her estate—the Messrs. Thomas Steer, Arthur Allen and Malcolm E. Reed—for their generosity in making this investigation and its continuance possible.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

1 to 8 Untouched photographs showing intranuclear crystals in hepatic cells of the Dalmatian dog. Approximately $\times 2600$.

1 to 4 Faces of crystals of different sizes. Figure 4, a bent crystal. Compare sizes of crystals with those of normal nuclei.

5 Faces of two crystals in the same intranuclear vacuole.

6 Transverse fracture of a crystal.

7 and 8 Several crystals—both faces and cross sections in single nuclei.

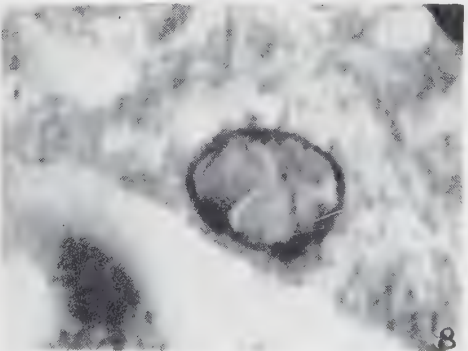
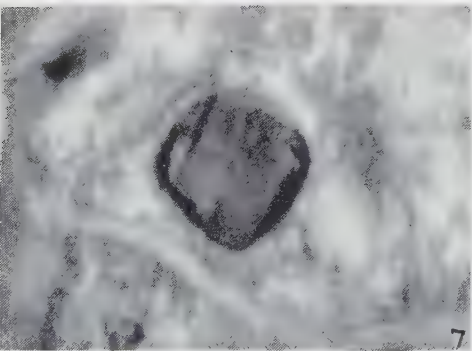
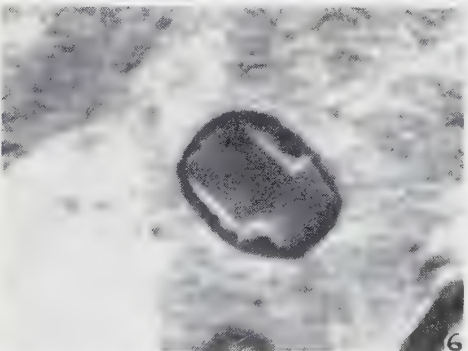
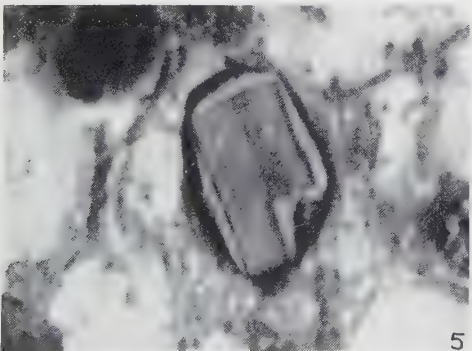
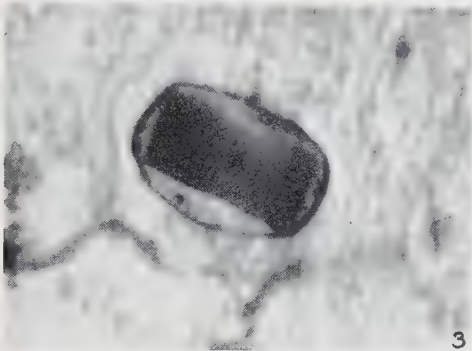
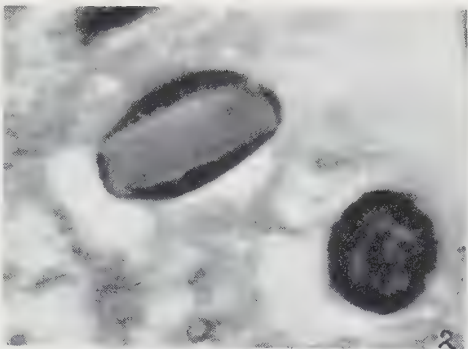
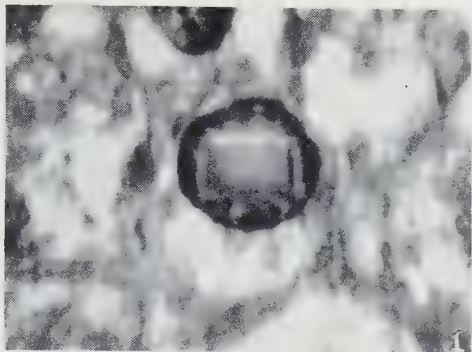
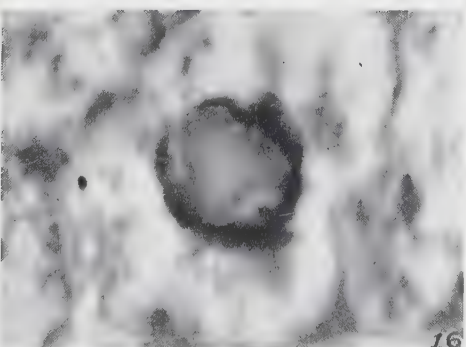
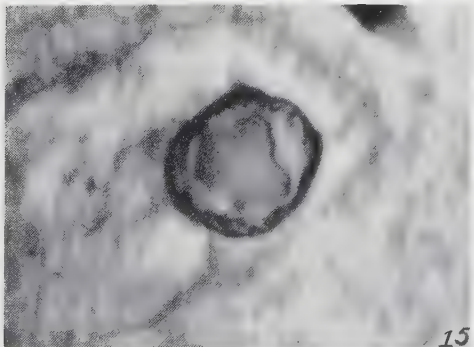
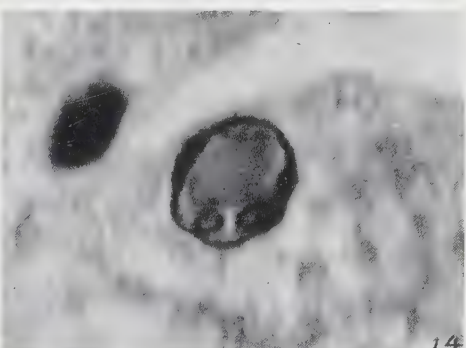
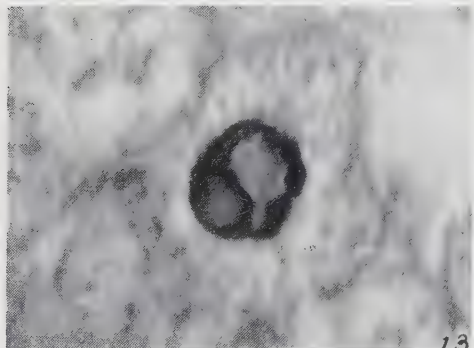
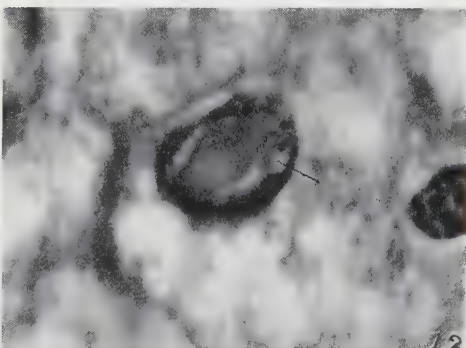
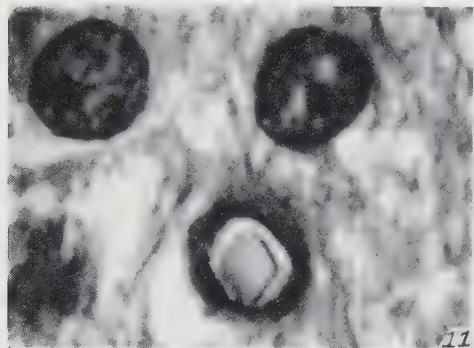
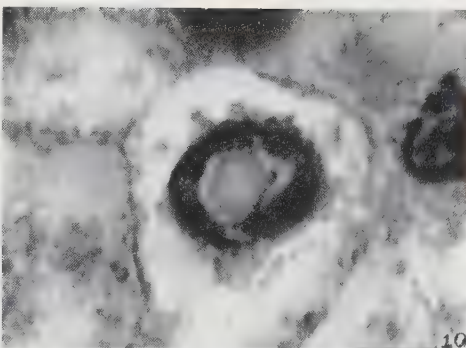
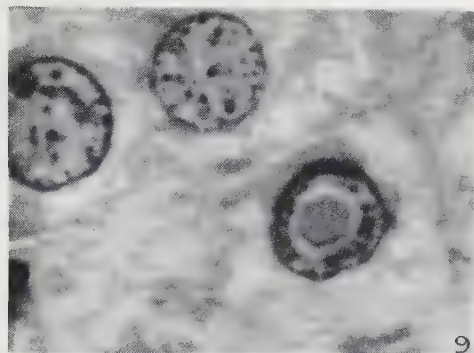


PLATE 2

EXPLANATION OF FIGURES

- 9 to 16 Untouched photographs—approximately $\times 2600$.
9 and 10 Hexagonal cross sections of typical intranuclear crystals.
11 Cross section—through cleavage two of the opposite faces are narrowed and the regular hexagonal form is modified.
12 to 14 Vacuoles containing amorphous masses in addition to those containing intranuclear crystals.
15 and 16 Cross sections of irregular, “twin” and “triplet” crystals.



SOME EFFECTS OF FIXATION AND OTHER INSULTS ON UTERINE EPITHELIAL CELLS IN PRIMATES

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TWO PLATES (TWELVE FIGURES)

The cytology of the uterine mucosa begins with the 1908 paper of Hitschmann and Adler. They described the progressive changes in epithelium and stroma during the menstrual cycle, using a variety of cytologic fixatives. The staining was largely with hematoxylin and eosin so the various organelles of the cell were not differentiated. They confirmed the fact that both surface and glands are lined by simple columnar epithelium in which some ciliated cells occur either singly or in groups. The enlarged cells of the "premenstrual" epithelium were found to have "club-shaped" enlargements. They were inclined to believe that such protrusions were due to post-vital changes. They definitely branded as an artefact the apparent merging of the cytoplasm of gland cells with the secretion in the lumen.

The fresh uterine epithelium had been studied earlier but for the most part before the days of isotonic media. The only figure of unfixed uterine cells seems to be Kölliker's (1854) figure 332 showing a cell "swollen with water." Subsequently attention was focussed on the presence or absence of cilia, and we have no report on the appearance of unfixed cells to serve as a control for the inevitable artefacts of preserved material.

The only cytological descriptions of uterine gland cells we have had are based on human material and they vary greatly, especially as to the appearance in the pro gravid stage. This in itself suggests that the cells are difficult to preserve at the

height of their activity. Schroeder ('09-'30), who studied preparations fixed in alcohol and stained with mucicarmine, added nothing to Hitschmann and Adler so far as cell form is concerned. He, too, described cells merging with the secretion in the lumen. He was the first to recognize the appearance of basal vacuoles as marking the beginning of his "secretory" phase (compare Möllendorff Handbuch, vol. VII, p. 456). Geist ('13), using formol or formol-Müller for fixation, described cells of the "late interval" and subsequent stages as characterized by large tongue-like processes projecting beyond the terminal bars. Gobert ('27), who paid particular attention to the matter of fixation, concluded that such "blebs" were artefacts as they were not present in her best material. Bartelmez and Bensley ('32) pointed out that protrusions beyond the terminal bars are useful as indices of cellular activity, whether they are artefacts or not, i.e., they present what Nissl in nerve cells called an "Equivalentbild." On the basis of the form and staining properties of the blebs they distinguished between two types of secretory activity. In addition they observed "brush borders" on the surface and gland cells in progravid stages. It is hardly necessary to say that such observations can be evaluated only in the light of extended observations on living or at least surviving cells.

Some preliminary observations on various mammals showed that the behavior of the uterine gland cells in the surviving condition varies according to the species. In connection with the work of Rossman ('40) an abundance of macaque tissue from various phases of the menstrual cycle was available immediately after removal at operation. A few similar studies on human material leave no doubt but that the conditions in these two species are substantially similar. I am indebted to Dr. Eva Tysse, Miss C. M. Bensley, and especially Dr. I. Rossman for help with preparation of the macaque material, and to Drs. Cary Culbertson and J. I. Brewer for the opportunity to study fresh human endometrium. Dr. N. L. Hoerr prepared the frozen-dried material using his isopentane freezing procedure.

The conditions under which fresh material is prepared play an important role in the appearance of the cells. The present macaque observations were made on wedges of uterine wall removed aseptically at laparotomy under nembutal (Abbott), usually without any clamping of the uterine vessels. Material from fifty-six cases was available. The first bits of tissue were under the microscope in 3 to 5 minutes after removal. Various neutral media were employed; the most satisfactory was autogenous blood serum, so far as one can judge from the delayed appearance of form changes and the persistence of active ciliary movement. Homogenous blood serum was practically as good; for vital dyes, aerated saline solutions were used. The postvital changes were found to be accelerated on the warm stage and so most tissues were studied at room temperature. In one such case cilia were still beating in an autogenous serum preparation 17 hours after the biopsy and at this time stroma cells had begun to migrate from the tissue fragment. Of the various vital dyes only Janus green B was used regularly in order to differentiate mitochondria from other granules; neutral red, Nile-blue sulfate and alizarine were used in some cases. Neutral red was segregated in vacuoles of various sizes, irregularly dispersed through the cytoplasm.

The fresh uterine mucosa has a mucilaginous consistency and sufficiently thin preparations could be obtained only by snipping off minute wedges with fine spring scissors after orienting the tissue under the binocular dissector. The instruments were coated with vaseline to reduce possible damage from electrolysis. The tissue was mounted so as to include some air bells and, after ringing with vaseline, studied with a 3.0 mm. 1.40 N. A. apo. immersion lens. Similar bits were left in Janus green for 5 to 15 minutes and studied in saline. The stages of the uterine cycle represented are as follows:

- Follicular phase—3 cases.
- Early lutein phase—16 cases.
- Late lutein phase—5 cases.
- Ovulatory menstruation—3 cases.

Anovulatory menstruation—1 case.

Post menstrual phase—1 case.

Early prograavid phase induced with estrin and progesterone—18 cases.

Late prograavid phase induced with estrin and progesterone—9 cases.

In all cases some of the material was prepared for sectioning. Nine additional specimens were available for histologic study.

If the tissue is observed promptly enough after removal, the contours of the epithelia are always smooth and clearly defined. This is the condition regularly seen in sections of "inactive" cells after prompt fixation. In fresh material it holds for all the stages examined, including prograavid uteri in which there is a maximum storage of secretion. The following observations deal only with cells that had not been injured mechanically. Injured cells, as Geist ('13) pointed out, immediately undergo pyknosis and shrinkage. In secretion-laden cells, 10 minutes after the removal of the tissue from the animal, some cells begin to bulge at the surface; occasionally every cell in a field will develop a "dome" that projects beyond the terminal bars. No further change may be visible for many minutes, but usually the domes enlarge rapidly producing characteristic tongue-like projections or "blebs" which appear clear and structureless. In the course of a few minutes they may become as large as the entire cell was originally. In form they resemble the blebs often seen in sections of human and macaque mucosae. No drawings or photographs of the fresh cells were made because all the pictures they presented could readily be matched in fixed and sectioned preparations. The clear blebs shown in figure 7 had already formed before fixation and they are realistic presentments of the large blebs seen in fresh preparations. The clear homogeneous exudate is well differentiated from the cytoplasm which is laden with mitochondria and spherules. There is much variability as to the length of time that may elapse before the blebs appear (5 to 40 minutes). As they develop, neither the nucleus nor

mitochondria change their position perceptibly, nor does the rest of the cell appear to change in volume.

When the blebs form rapidly they promptly disintegrate. This may result in a hazy cell border, but usually the contour again becomes sharp and the only remaining traces of the spectacular bleb formation are the large globules of secretion on the surface of the epithelium.

All cells do not necessarily develop blebs; whether they do or not, other changes soon become visible. Thus spherules may appear in the cytoplasm (compare fig. 2). These are usually larger than mitochondria and they have been recognized only in the zone between nucleus and lumen, whereas mitochondria are abundant basally as well as superficially (Bartelmez and Bensley, '32). The spherules are variable in size; the smallest are not much larger than the granular mitochondria but can be differentiated from them in that they do not stain with Janus green. More significant is the fact that they are not visible until several minutes after the preparation has been mounted.

The question that arises immediately is, "are they secretion granules," i.e., are they characteristic phases in the segregation of a specific component of the uterine secretion? They usually enlarge promptly and may aggregate at the surface of the cell which then appears vacuolated. Under these conditions there may be no cellular protrusion. The clear ends of the cells at ↓ in figures 4 and 5 exhibit the superficial vacuolation produced by the enlargement of the granules. The evidence that these granules and vacuoles are artefacts is summarized on pages 519, 520.

In a few cases a faintly striated border was seen to form on the surface of the epithelium. It was not interrupted by terminal bars but appeared continuous like the intestinal brush border (compare p. 520 and + in fig. 5). In uterine epithelium it is manifestly an artefact like dome and bleb formation.

It is rather difficult to obtain a gland favorable for observation and the gland cells could be carefully studied in only seventeen cases. It is clear, however, that the various post-

vital changes always appear sooner in the surface than in the glandular epithelium. Blebs often appear on gland cells; usually 5 to 30 minutes later than on the surface. The simplest explanation of this is that the film of secretion on the surface epithelium is easily washed off and the cells are consequently brought into intimate contact with the mounting medium, which though isotonic is nevertheless an alien environment. The gland cells, on the other hand, are protected by the glairy secretion which fills the lumen. If one can judge from the very superficial penetration of highly dispersed dyes like neutral red, the ions of the mounting medium do not enter rapidly into the substance of a mounted tissue fragment. If this interpretation is correct, bleb formation and the like are not due to anoxemia, since in an aerated medium the glands in the interior of a tissue fragment have less available oxygen than those on the surface. This is clearly indicated by the more rapid reduction of vital dyes in the interior of a tissue fragment and by the fact that the changes are not as rapid in the unopened extirpated uterus as they are in tissue fragments in a dish of serum with ready access to air.

Blebs develop on some of the cells in specimens removed at every stage of the cycle. They appear even in mucosae usually described as "inactive," i.e., associated with ovaries that have only small follicles. The changes are more rapid and appear in a larger proportion of cells when the store of intracellular secretion is greatest, that is, in late progravid and early gravid phases. Since typical blebs have been seen to develop in specimens that had no glycogen (as was shown by appropriate methods in the sectioned material), it seems probable that they are due to the presence of another component of the uterine secretion. The process is obviously akin to the explosion of many types of mucus-secreting cells (compare Heidenhain, '11). All the changes observed may be attributed to the imbibition of water by a hygroscopic intracellular secretion antecedent. The question is: Does the discharge of secretion normally involve abrupt changes in cell form, or does the secretion ooze gradually from the surface?

The latter is definitely indicated by the fact that the secreting cells were uniform in shape and the contours of the surface and glandular epithelia were smooth and sharply defined in every specimen observed within 3 minutes after removal. Since the series includes many specimens in maximal secretory activity, we are justified in concluding that any protrusion beyond the terminal bars is an artefact. This is confirmed by the study of frozen-dried and promptly fixed tissues.

SOME EFFECTS OF FIXATIVES ON FRESH PREPARATIONS

If a fresh mount under the microscope is irrigated with fixing fluid, various changes may be observed. They are influenced largely by the "age" of the preparation, i.e., the time that has elapsed since removal from the animal. The particular fixing fluid used also plays a part. If the material has been mounted for only 1 or 2 minutes and is then irrigated with aqueous chrom-sublimate-formol (the "formol-Zenker" of Bensley), the cells become dull and opaque without being visibly altered in form. The mitochondria previously seen can still be recognized. The cells resemble those seen in sections from the surface of blocks fixed in less than a minute after the blood has ceased to flow through the tissue. If Bouin's fluid or acetic acid in a concentration of 2% or more be used, there is likewise no change in form but the cytoplasm "clears" and the mitochondria disappear from view. If, on the other hand, some time has elapsed since preparing the mount, one sees striking changes the moment a colored fixative reaches the cells in sufficient concentration to be visible. Some specific cases will illustrate this:

No. 223b. (Progravid stage, see fig. 8.) The mucosa was observed in aerated Ringer's solution, 20 minutes after removal from the animal. A gland was found as yet free from blebs and during the following 12 minutes none appeared. Then dilute acetic acid (ca. 0.5%) was run under the coverslip. The effect was instantaneous and spectacular. Great blebs ballooned out from some cells while others seemed to "ex-

plode," becoming spheroidal. In fact, many of the cell pictures appeared which are so characteristic of sections of the human decidua vera.

No. 233b. (Early pro gravid stage.) This was a preparation like the preceding in which, however, large blebs had already formed. When the fixative (formol-Zenker in this case) reached them, the blebs separated from the cells. This corresponds to the apparent merging of cells and secretion often seen in poorly preserved material.

No. 227c. (Early pro gravid stage.) An area of surface epithelium was being studied in which no blebs had appeared. Twenty-six minutes after removal from the animal the preparation was irrigated with formol bichromate. As the first tinge of yellow became visible every cell threw out a great clear bleb, duplicating the picture shown in figure 7. The formation of the bleb was too rapid to be followed by the eye.

No. 237b. (Late pro gravid stage.) A bit of tissue was removed and placed immediately in a 5% solution of chloral hydrate in Ringer's solution. In 25 minutes there were exceptionally large blebs on all the epithelial cells whereas they began to form only after 35 minutes in the tissue kept in autogenous serum but not mounted on a slide. Chloral hydrate appears to accelerate rather than retard the appearance of this particular artefact.

SOME ARTEFACTS OF PREPARED SECTIONS

The observations recorded above were made on transparent bits of tissue in isotonic media. When a small block of material, 2-3 mm. thick, is fixed promptly, the conditions are quite different because it takes a certain amount of time for any fluid to penetrate to the interior. During this interval the cells are undergoing changes which become manifest when the fixative reaches them in effective concentration. In considering the effects of fixatives it is important then to take into consideration the location of a given cell with reference to the surface of the block (compare Underhill, '32). This is the basis for most of the differences between surface and glandular epithelium seen in sectioned material.

Cytological preparations were made from all the cases studied supravitaly and from several others. Bits of tissue or larger pieces were fixed in various fluids at various times after removal. This material was dehydrated with alcohol, usually double imbedded in celloidin-paraffin, cut 3 to 5 μ thick and stained in various ways. The pictures presented in such sections could be evaluated in the light of the fresh preparations and compared with one another. The principal conclusion from such study is that the uterine epithelial cells are exceptionally difficult to fix and that certain changes occur during the interval between the cessation of blood flow through the mucosa and the arrival of the fixative diluted with tissue juices in a concentration sufficient to coagulate the proteins. This may be only a matter of seconds; in any case, less time is involved than in the appearance of artefacts in fresh preparations. In other words the fixation artefacts are produced in cells which if studied fresh would not yet have exhibited any visible changes. This is confirmed by the finding that a delay of several minutes prior to freezing (in preparation for the Altmann-Gersh method) does not involve the changes in cell form which are invariably seen in fixed material at the center of a block of tissue. We shall consider certain of these fixation artefacts in the order of their appearance. "Delay" in fixation refers either to delay in immersing the block in the fixative or to the delay involved in the penetration of the fixing fluid to the cells in question.

A. Glycogen artefacts. When glycogen is abundant in epithelial cells it can only rarely be prevented from plasmolyzing during fixation. If one employs formol-bichromate or aqueous chrome-sublimate-formol and the fixation is prompt, most of the glycogen can usually be preserved (C. M. Bensley, '39), and by this means the characteristic distortions of alcohol fixation, as shown in figure 12, can be minimized. Nevertheless, even if small fragments of pro gravid macaque mucosa be fixed within 15 seconds after the uterus has been incised, the great majority of cells have the glycogen segregated in

large discrete vacuoles such as are never seen in fresh material (figs. 3, 4, and 5).

The glycogen seems to be unusually labile in late follicular and early lutein phases both in *Homo* and *Macacus* and appears to separate out with water as the protoplasm is coagulated. Thus large vacuoles are formed which in the sections can be shown to have glycogen precipitated over their walls. If the glycogen has not been preserved or has not been stained, the vacuoles appear empty. They can be used as indicators of the presence of intracellular glycogen since they are never present except when it is abundant. On the other hand glycogen may be present in cells which have no vacuoles. This is especially true of human material. Fat spherules when they occur in the epithelial cells, are much smaller than the typical glycogen vacuoles. As may be seen at ↓ in figure 8, the vacuoles are unstained in mucicarmine preparations after formol Zenker fixation. The evidence that they are artefacts is as follows:

1. Fresh material from the specimen illustrated in figure 3 was studied for $1\frac{1}{2}$ hours after removal but showed no sign of large vacuoles. This is not surprising as glycogen is promptly hydrolyzed postvitally to glucose. The cells illustrated were in the fixing fluid 30 seconds after the uterus was incised. All ordinary fixatives plasmolyze intracellular glycogen when it is present in abundance, producing similar or still more blatant artefacts.

2. Material prepared by the freezing-drying technique of Altmann-Gersh may be frozen solid within 2 to 3 seconds after removal and in it the vacuoles are absent and glycogen frequently appears uniformly distributed. When it is localized it appears diffused over a restricted area of cytoplasm and not segregated so that its solution could produce a vacuole. This is also true of material fixed in warm osmium tetroxide vapor (compare Rossman, '40).

3. The more violent the plasmolysis the more extreme is the distortion of the glycogen picture. Thus when strong alcoholic fixatives are used the glycogen is driven before the incoming solution and precipitated against the distal cell walls

so that it may be basal on one side of a gland, superficial on the other. Nuclear chromatin may be similarly plasmolyzed under these conditions (fig. 12 ↓).

B. Golgi apparatus. In cells laden with secretion one sees a more subtle result of the slow penetration of sublimate fixatives into tissue. This is a change in the form of the Golgi apparatus. In material fixed within 30 seconds after removal, the Golgi apparatus in the superficial cells appears in the form of irregular strands or nets rather widely dispersed in the cytoplasm between nucleus and surface. As one passes deeper into the block the strands become more closely aggregated. Still deeper in, they cannot be recognized at all and one sees the picture shown in figure 6 at ↓ where there is simply a spheroidal "paranucleus" which stains deeply in hematoxylin solutions.

C. Secretion granules and vacuoles. The "secretion" granules that become visible in fresh material some time after mounting (p. 513) can be found after fixation by various methods and even in tissue dropped into the killing fluid a minute or two after the blood has ceased to flow through it (fig. 2). In such cases, however, the granules are not found superficially but appear as one passes on toward the interior of the block. Similarly they do not appear if fixation occurs 15 to 20 seconds after incising the uterus. They are present even on the surface of a block if there has been a delay of fixation for 10 minutes or more. This also indicates that they are not present in the living cell but appear de novo as a result of the changes in the cellular environment. They neither stain supravitaly with Janus green (p. 513) nor red in Altmann mitochondrial preparations. Furthermore one can readily find a series of transitions between minute granules of the same order of magnitude as granular mitochondria and the clear vacuoles which may fill the superficial end of the cell (at ↓, figs. 4 and 5). This parallels the enlargement of the granules into vacuoles observed in supravital preparations and appears to be due to the intake of water by a hygroscopic substance. The fine granules of this type appear as minute

dark blue granules after the azan-Mallory technique of Heidenhain which stains the mitochondria red or leaves them unstained (fig. 2 at +). In paraffin sections the "secretion" granules are surrounded by a halo which suggests differential shrinkage as compared to the surrounding cytoplasm. They are always superficial to the nucleus; frequently they are grouped near the lumen where they may be arranged in one or more rows under the limiting membrane. They are variable in size in fixed material. The larger they are the more they tend to be stained orange or pink in azan-Mallory preparations. When the vacuoles aggregate at the lumen they may stain with mucicarmine and the cells come to resemble the "theca" cells of the gastric foveolae which are likewise fixation artefacts. From this type of cell with a "theca" we pass over directly to the "exploded" cells described on page 522.

D. Changes in cell form: 1. "Brush borders." Under the best conditions of fixation all the epithelial margins are as smooth as in a newly mounted fresh preparation (compare right side of fig. 1). As in figure 3 this is usually obscured by the film of secretion precipitated on the surface by the fixative. Even in promptly fixed material some cells may have a "brush border" due to the formation of threads of secretion extending from the surface (+ in fig. 5). They may be loosely spaced as in this figure or closely crowded and they may vary in thickness from cell to cell. Since they have been seen in fresh preparations, they cannot be mere coagulation pictures. They seem to represent an outpouring of secretion in fine streams from the surface of the cell. They do not move *in vivo* and never attain the length of cilia which in this material always have basal bodies (compare fig. 1 +).

2. Domes and blebs. If a minute or more elapses between the removal of a block and fixation, the epithelial borders of active cells appear uneven, and the cell membranes bulge beyond the terminal bars to form domes and blebs. Figures 6 to 11 show all transitions from a slight rounding of the surface (* in figs. 8 and 9) to the great protruding tongue at ↓ in figure 10.

The blebs may be clear and structureless like those seen in post vital preparations (fig. 7). Usually they show a gushing out of the cytoplasm as at ↓ in figure 10 and in figure 11. Such blebs stain as deeply as the rest of the cytoplasm and may contain mitochondria. In the cell indicated by the arrow (↓) in figure 10 a clear bleb formed first and then the cell "exploded" producing an underlying more deeply stained protrusion. Some cells react only to the extent of enlarging somewhat like those indicated by arrows (↓) in figures 8 and 9. Since blebs and the other forms of cell explosion have been seen to form in fresh material under the cover slip as the concentration of the fixative was increasing (p. 515) it would seem probable that certain changes begin as soon as the blood flow ceases but they do not become manifest until the process of coagulation begins. The longer the delay and the more secretion that has been accumulated in the cell the more it is distorted. When there is not much secretion and the cells are narrow, the blebs remain small and clear ("type I secretion" of Bartelmez and Bensley). When the cells are heavily laden it is very difficult to prevent the formation of large deeply staining protrusions ("type II") especially in the deeper parts of the block which are not reached by the fixative for minutes or longer after immersion (compare Underhill, '32; Baker, '33). They may even be present at the center of small fragments which have been dropped into rapidly penetrating fluids like Bouin's within 15 seconds after incising the uterus. When blebs are shrivelled up in the course of preparation the familiar "frayed" appearance is produced.

Blebs or other cell distortions are present in practically all sectioned material and their size in promptly fixed material can be used as an index of the amount of accumulated intracellular secretion. They are usually largest in the earlier gravid and late progravid stages (p. 514) but it must be remembered that cells from different stages of the cycle cannot be compared unless they have been fixed equally promptly and lie at comparable distances from the surface of the blocks.

3. "Explosions." Sometimes, especially at the height of secretion storage, many cells undergo another change during fixation. This involves a general increase in size without the formation of any marked protrusions from the surface.

This change has not been seen in surviving cells kept in isotonic solutions but it is a striking phenomenon when such fresh preparations are irrigated with fixatives, especially those containing acetic acid (p. 515). The cells indicated by arrows (↓) in figures 8 and 9 are mild expressions of this artefact. The mucicarmine preparation (fig. 8) shows that the secretion in such cells gives the reaction for mucus. It will be recalled that if the fixation is sufficiently prompt the intracellular secretion does not stain with mucicarmine.

SUMMARY

Uterine epithelial cells in *Macacus* and *Homo* quickly respond to alterations in their environment by changes in form or in the state of the stored secretion antecedents. Studied supravitaly in appropriate media they soon change as a result of general osmotic disturbances rather than anoxemia (compare p. 514). The epithelial borders are smooth and discrete in all phases of the reproductive cycle. Within 10 minutes after mounting a fresh preparation there may be a rapid extrusion of clear blebs (compare fig. 7) or the segregation of mucoid secretion in the form of spherules (fig. 2) which enlarge to form vacuoles and segregate at the surface (figs. 4 and 5).

Unaltered cells may develop blebs or may "explode" when a fresh preparation is irrigated with certain fixatives.

Sections prepared by standard methods show certain artefacts when the cells are loaded with secretion. If the fixation is prompt the artefacts can be used to identify active cells.

The following artefacts are described: 1. Large vacuoles resulting from the segregation of glycogen during fixation (figs. 3, 4, and 5). Glycogen may, however, be present in a form that does not produce vacuoles. This is particularly true of the late progravid and early gravid phases in the human endometrium. 2. An abundance of the mucoid component of

the secretion is indicated by postmortem increases in the volume of the cytoplasm. All transitions are seen from slight doming of the surface (* in fig. 8) to large tongue-like protrusions (figs. 10 and 11). Other cells show a general enlargement (↓ in figs. 8 and 9). This may result in pressure on neighboring cells which become pyknotic "rod" cells (+ in fig. 8).

There are evidences that the dispersed Golgi net which is seen in cells at the surface of blocks fixed in sublimate solutions, becomes concentrated toward the interior, so that the inner cells show only a deeply stained area ("paranucleus") adjoining the nucleus (↓ in fig. 6).

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EXPLANATION OF PLATES

The figures are all from untouched photomicrographs taken with a 2.0 mm. apochr. imm. lens and $\times 10$ ocular at 800 diameters magnification and enlarged to 1200 diameters. The artefacts shown on plate 1 are characteristic of promptly fixed cells, those of plate 2 indicate several minutes delay either in immersing the tissue in the fixative or in the penetration of the fluid into the block.

PLATE 1

EXPLANATION OF FIGURES

1 Surface epithelium from a biopsy specimen removed on the nineteenth day of the cycle about 9 days after ovulation. In fresh material the outer contour is always more smooth than after fixation; this is the best that can be done with fixed cells during the phase of active secretion. There is a fine precipitate on the surface of the cells to the left. +, ciliated cells. Blebs were slow to form in this material when studied supravitally. Figures 2 and 11 are taken from the same block. Fixed in aqueous chrome-sublimate 1 minute after opening uterus, cut at $3\ \mu$ and stained with Mallory azan (226b).

2 From the same section as figure 1. Glandular epithelium about 0.6 mm. from the surface of the block. High focus to show granular secretion aggregates at + (compare fig. 11). These granules are slightly larger than granular mitochondria; all stages in their transformation into secretion vacuoles can be followed both in supravital preparations and in promptly fixed material, where the vacuolated end of the cell resembles the "thecae" shown in figures 4 and 5 at $\downarrow$. The surface is covered by a delicate brush border faintly visible in the figure. Photomicrograph by R. D. Bensley.

3 Epithelium from a gland 0.4 mm. from the surface of a block fixed 30 seconds after removal at biopsy on the twenty-third day of a cycle, 8-10 days after ovulation. The surface contour is even, though obscured by coagulated secretion. The glycogen has been segregated during fixation in large sharply delimited vacuoles basal to the nucleus. Compare the negative picture of the Golgi apparatus in the cells at $\downarrow$ with that indicated by $\downarrow$ in figure 6. Aqueous formol-chrome-sublimate osmic (Maximow) $4\ \mu$ paraffin section stained with phosphotungstic acid hematoxylin (231a).

4 From a gland near the surface of a specimen removed on day 27 of a cycle, 10-11 days after ovulation. The tall pseudostratified epithelium is the usual type in both surface and glands of this specimen. The cells indicated by the arrows ($\downarrow$) are seen to extend through the thickness of the epithelium. The glycogen vacuoles are usually but not always basal. Fixed in aqueous chrome sublimate, cut at $3\ \mu$ and stained with Mallory's phosphotungstic acid hematoxylin (211).

5 Glandular epithelium about 0.5 mm. from the surface of the same block as shown in figure 4. Blebs appeared promptly in the fresh preparations but in this block the artefact was avoided by prompt fixation. Three other artefacts are to be seen: the brush border (at +), "thecae" at $\downarrow$, and large glycogen vacuoles above and/or below the nucleus. Photomicrograph by R. D. Bensley.

6 Epithelium of gland from a block removed on day 23 of an ovulatory cycle (5 days after trauma to uterus). There was but little glycogen in this mucosa and no vacuoles have formed but several minutes delay in fixation has resulted in the appearance of domes (at +) and the concentration of the Golgi area into a deeply stained mass superficial to the nucleus (at $\downarrow$). Zenker stock fixation, cut at $6\ \mu$, iron hematoxylin (217b). Preparation by I. Rossman.

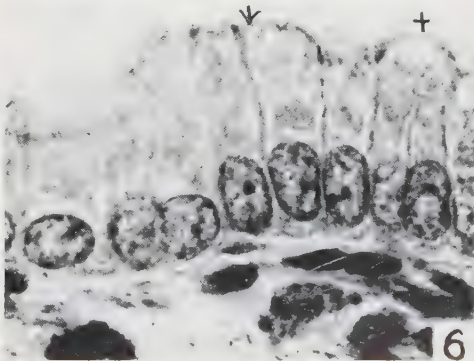
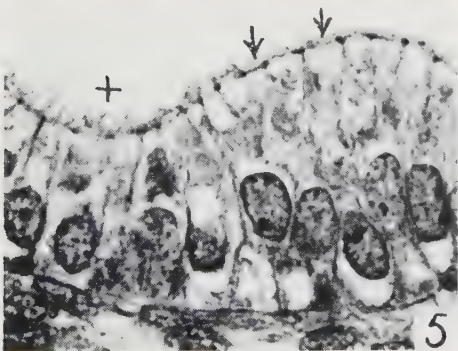
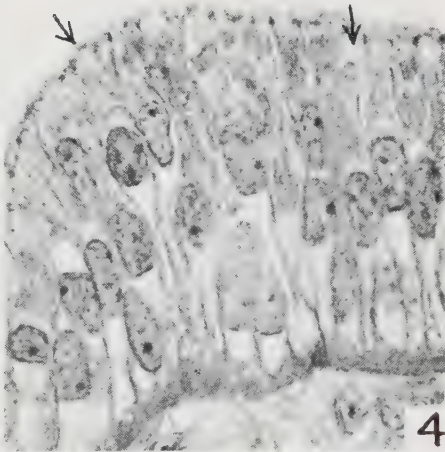
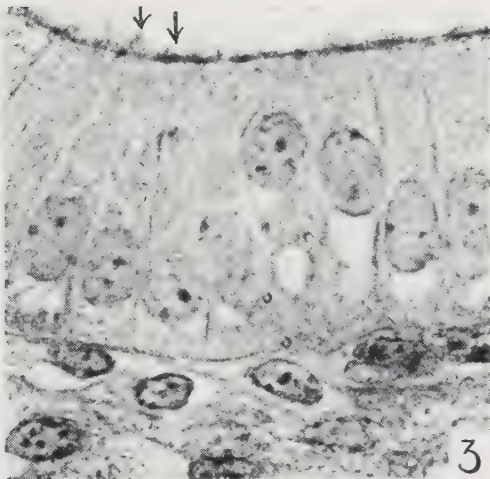
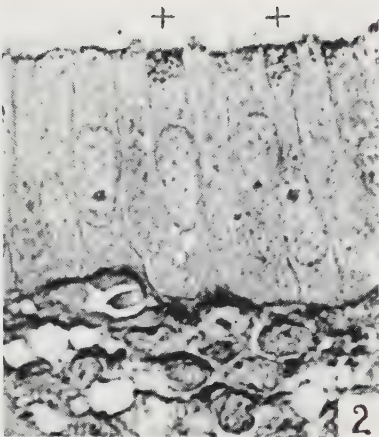
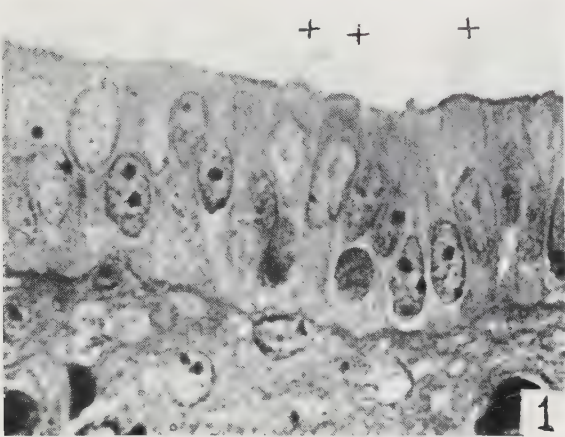


PLATE 2

EXPLANATION OF FIGURES

7 Complete vasoligation of uterus, 5 hours before hysterectomy on twenty-fourth day of an anovulatory cycle. Every cell has a clear vesicle extending beyond the terminal bars. These blebs closely resemble those which can be seen arising in fresh preparations (compare p. 512). Stroma cells and fibers have autolyzed. Aqueous chrome-sublimate, 3μ section stained with phosphotungstic acid hematoxylin (210 C).

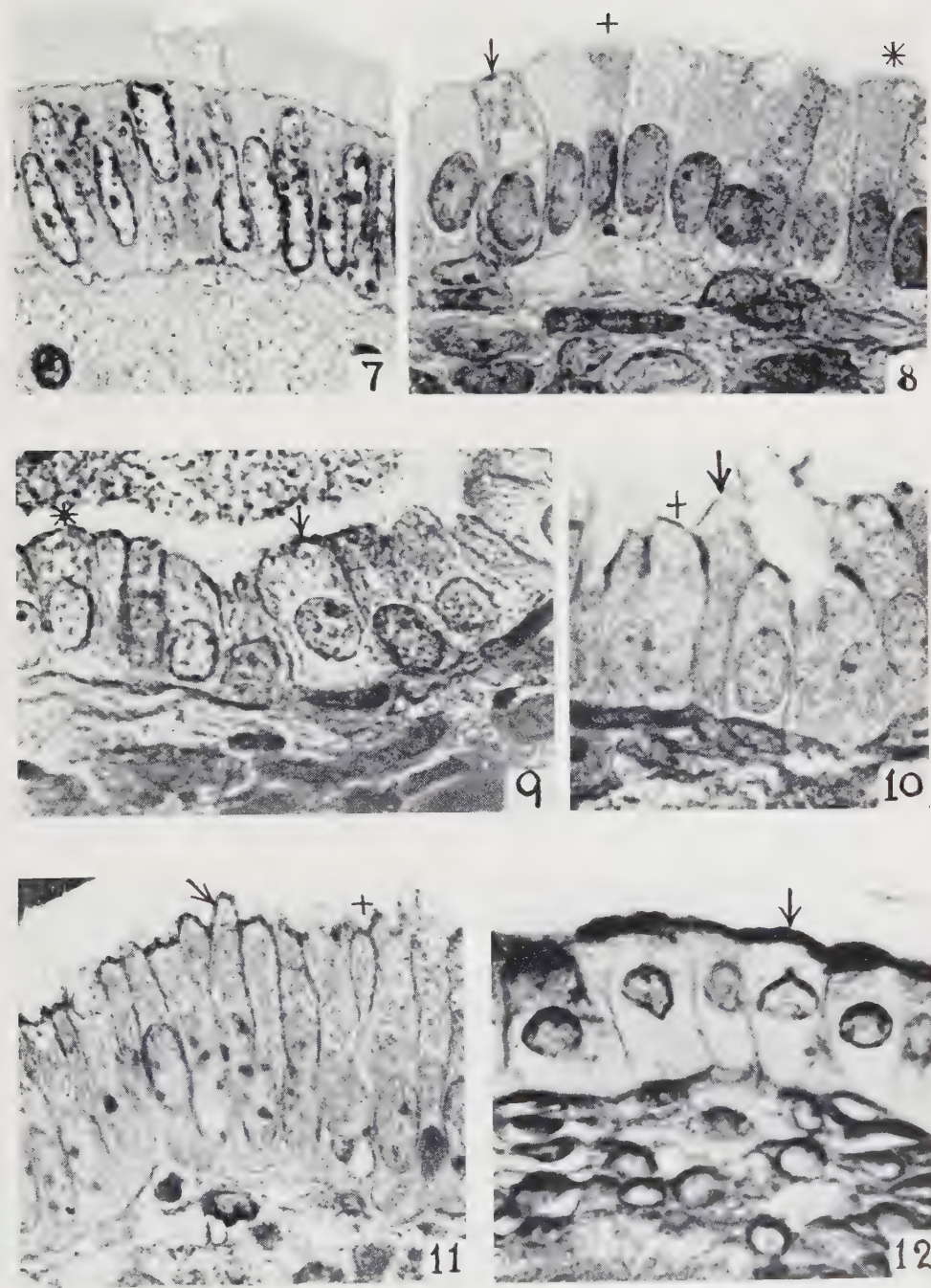
8 Cells near the mouth of a gland fixed 5 minutes after removal from the animal, show various artefacts due to the delay. Twenty-third day of an experimental (oestrin-progesterone) cycle. The cell indicated by the arrow ($\downarrow$) had begun to explode and its cytoplasm stains with mucicarmine (compare p. 522). The vacuoles contain glycogen. The cell at * shows early dome formation. The cell at + shows signs of being compressed by the enlargement of its neighbors during fixation. All transitions from this to typical rod ("peg") cells can be found. Fixed in aqueous formol-chrome-sublimate, cut at 3μ and stained with mucicarmine and hematoxylin (223b).

9 Gland cells at the surface of a block fixed 4 to 5 minutes after removal on the thirty-first day of an experimental (oestrin-progesterone) cycle. The cell at $\downarrow$ shows a general enlargement of the cytoplasm without bleb formation (compare $\downarrow$ in fig. 8). At * doming and glycogen vacuole formation are beginning. Aqueous formol-chrome-sublimate, 4μ section Mallory azan (221b).

10 Gland cells removed on day 38 of an experimental (oestrin-progesterone) cycle, comparable to a 16-day pregnancy. The block was fixed in aqueous formol-chrome-sublimate, 3 to 4 minutes after hysterectomy. The cells show the effects of this delay as well as the delay due to the penetration of the fixing fluid. All of them have developed larger or smaller blebs extending beyond the terminal bars. At $\downarrow$ the cell seems to have had a clear bleb like those of figure 7 and then the cytoplasm blew up producing the deeply stained protrusion so characteristic of sections of late progravid or pregnancy glands. Typical fixation bleb at + (212d).

11 Epithelium from a superficial gland of the same nineteenth day specimen illustrated in figures 1 and 2. The secretion aggregates are present here also (at +) and in addition many cells have developed blebs ($\downarrow$) which contain granular secretion.

12 Glandular epithelium showing plasmolysis produced by violent fixation. In the cell at $\downarrow$ not only the glycogen (deeply stained) has been displaced to the surface, but the chromatin has been driven against the nuclear membrane distally. Frozen dried material from this specimen shows the glycogen widely and often uniformly dispersed through the cytoplasm. From a 17-day deciduoma prepared by I. Rossman. Fixed in formol-pierce-alcohol and stained by the Feulgen-Bauer method. Photomicrograph by R. D. Bensley.



DECREASE IN HUMAN NEURONES WITH AGE

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THREE FIGURES

In a previous study Corbin and Gardner ('37) found the number of fibers in human spinal roots to decrease 32% between the third and ninth decades. The study has been continued in order to obtain a larger series and to make a more accurate determination of the decrease. Surmising that decreases in the number of fibers in dorsal roots are due to a loss of spinal cells, these cells were counted in thirty-one bodies varying in age from 34 to 85 years.

MATERIALS AND METHODS

Portions of the eighth and ninth thoracic ventral and dorsal roots and the associated ganglia were removed from bodies preserved for dissection. All the roots were further fixed in Müller's fluid, dehydrated in dioxan, embedded in paraffin, sectioned at 10 μ , and stained with Mallory's mixture of aniline blue and orange G. As in the previous study the material was excellent and counting could be done without difficulty. Recounts made on seven dorsal and ventral roots showed less than a 5% variation between maximum and minimum. The actual percentage differences were 4.6, 2.5, 4.6, 2.7, 4.4, 2.2, 3.7, 1.6, 3.5, 3.7, 4.1, 2.4, 1.8, and 2.2, or an average difference of 3.14, which is equivalent to an average error of $\pm 1.6\%$.

The ganglia were treated in the same way and sectioned serially at 10 μ . Numerous stains were tried but most of them proved unsatisfactory because the nuclei contain little chromatin and the nucleoli failed to stain. However, a combination

of toluidin blue and aqueous eosin gives uniformly good results. This mixture stains the cytoplasm pale pink, the unsectioned nucleus a darker pink, and the nucleoli a bright, metallic blue. This gives a clear-cut differentiation and enables one to count nucleoli without any difficulty. The nucleoli in every fourth section were counted and the average count multiplied by the number of sections on a slide. The estimated total counts for each slide were then added to obtain the total number of cells within the ganglion. The results obtained by first counting every fourth section and then every section, showed a difference of approximately 3%. Nucleoli which were divided by the knife and appeared in more than one section, may have been a source of error, although according to Jones ('37) no account need be taken of this if the sections are cut at $10\ \mu$ and none but definite and distinct nucleoli are counted.

RESULTS

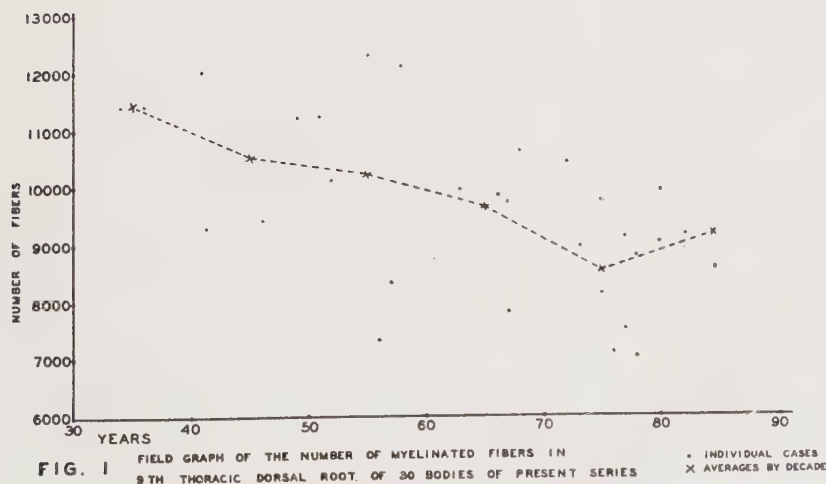
As in the previous study, considerable variation was found in the number of fibers in homologous dorsal and ventral roots within each decade. The ages, the recorded causes of death and the root counts for each body are given in table 1. Decade averages, computed for the ninth thoracic dorsal root, are represented graphically in figure 1, which indicates a gradual decline in the number of myelinated fibers from the fourth to the eighth decade, although a similar decrease is not apparent in the ninth decade. The four cases in the fifth decade show a 2.6% decrease from the two in the fourth decade, and the seven from the sixth decade show a further decrease totaling 6.5%. The five from the seventh decade show a decrease of 11.7%; the nine from the eighth decade show a decrease of 21.6%; and the four from the ninth decade show a decrease of 12.7%. This percentage decrease is less than that obtained in the previous series probably because no specimens were available in the second and third decades and the decrease was hence calculated from a peak taken later in the life span.

TABLE 1

Root fiber counts from 31 bodies and ganglion cell counts from 21 bodies

Body No.	Age	Cause of death	8T DR	8T VR	9T DR	9T VR	8T DRG	9T DRG
786	34	Pulmonary tbc.	10767	5546	11398	6038	31364	30195
789	36	Pulmonary tbc.	8563	6087	11393	5258	34300	38035
792	41	Cirrhosis of liver	9303	4897	9264	5097	37219	31671
798	41	General paralysis	11468	5826	12046	5839		
795	46	Cirrhosis of liver	9218	6226	9414	4967		
787	49	Chronic nephritis	10807	5491	11208	5658	41343	41081
797	51	Aortic insufficiency	10801	5408	11202	5241	37231	34128
827	52	Cirrhosis of liver	9711	5352	10072	5526	36959	37238
788	55	Skull fracture	11383	6593	12275	6146		30004
813	56	Arteriosclerosis	8671	5116	7320	4389	28618	29468
825	57	Arteriosclerosis	7769	4215	8273	4403		
816	58	Arteriosclerosis		4279		4567		
800	58	Apoplexy	11253	6075	12148	5600		32639
820	63	Cirrhosis of liver	8551	4637	9888	4985	30575	35205
818	66	Arteriosclerosis	9182	4846	9842	5080	19415	23403
822	67	Bronchopneumonia	6798	4455	7798	4876	25815	23686
805	67	Coronary occlusion	12121	4735	9739	4900	28939	26449
823	68	Arteriosclerosis	9321	5808	10636	5415	24596	26567
812	72	Arteriosclerosis	9132	5121	10365	5395		
814	73	Arteriosclerosis	8845	3978	8915	3703		28503
790	75	Chronic myocarditis	8264	4302	8109	4682	25412	24187
796	75	Chronic myocarditis	8927	4490	9685	4981		
817	76	Arteriosclerosis	7693	3599	7190	4093	25119	28391
829	77	Arteriosclerosis	6887	3845	7458	4149		
815	77	Arteriosclerosis	6770	4356	9067	5377		
821	78	Arteriosclerosis	8418	5703	6970	4735	22995	23176
819	78	Arteriosclerosis	9417	3598	8846	3376	30647	28702
794	80	Acute myocarditis	7757	4959	9885	6075	27690	32325
826	80	Arteriosclerosis	9189	5454	9027	5039		
802	82	Bronchopneumonia	10253	5501	9166	5185		
824	85	Arteriosclerosis	7225	4613	8621	5173	21044	24371

8T, 9T, indicate eighth and ninth thoracic
DR, VR, indicate dorsal and ventral roots
DRG, indicates dorsal root ganglion



The counts of the 1937 series were therefore combined with the present ones and the decade averages for each root and for all four roots computed. The results are shown in table 2. Figure 2 graphically represents the losses found in the ninth thoracic dorsal root. The highest average count is that in the second decade which is 187% of that in the 1-day-old infant, but practically the same as that in the third decade. The two cases in the fourth decade show a 7% decrease from the second. As shown in table 2, this decrease totaled 27% for the sixteen cases in the eighth decade, but only 21.6% for the five in the

TABLE 2

Averages by decades of root fiber counts of sixty-one bodies of the present and 1937 series

	NEW-BORN	10-19	20-29	30-39	40-49	50-59	60-69	70-79	80-89
8T DR	4178	10628	11058	9665	9368	8658	8169	7962	8286
8T VR	1777	5968	6205	5816	5495	5361	4805	4420	4923
9T DR	4365	12301	11304	11395	10320	9841	8522	8523	9134
9T VR	1881	6081	6204	5648	5506	5293	4845	4628	5086
Averages for 8th and 9th thoracic dorsal and ventral roots	3050	8744	8693	8131	7672	7288	6585	6383	6857
Per cent of increase over 1 day old		187%							
Per cent of decrease from the 10-19 year group			0.59%	7%	12.3%	16.7%	24.7%	27%	21.6%

ninth decade. The 27% decrease in the eighth decade is practically the same as that obtained in the 1937 series which was 32%. The decrease in the ninth decade is much less and in view of the individual variability it is regrettable that more cases from the decade were not available.

The ganglion cell counts with each decade represented also varied, but not as much as the root fiber counts, and the earlier decades were not represented. Decade averages computed for both ganglia are represented graphically in figure 3 which shows a rise in the number of cells, from the fourth to the

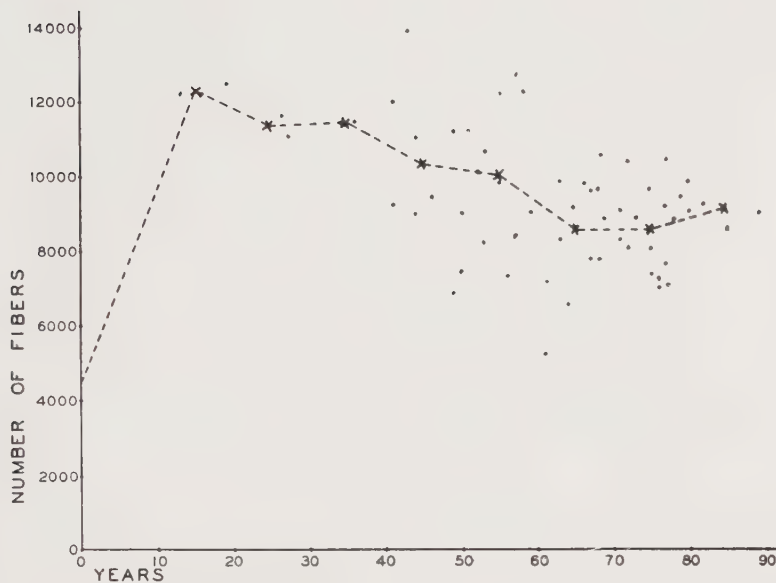


FIG. 2 FIELD GRAPH OF THE NUMBER OF MYELINATED FIBERS IN 9TH THORACIC DORSAL ROOT OF 62 BODIES OF '37 AND PRESENT SERIES.

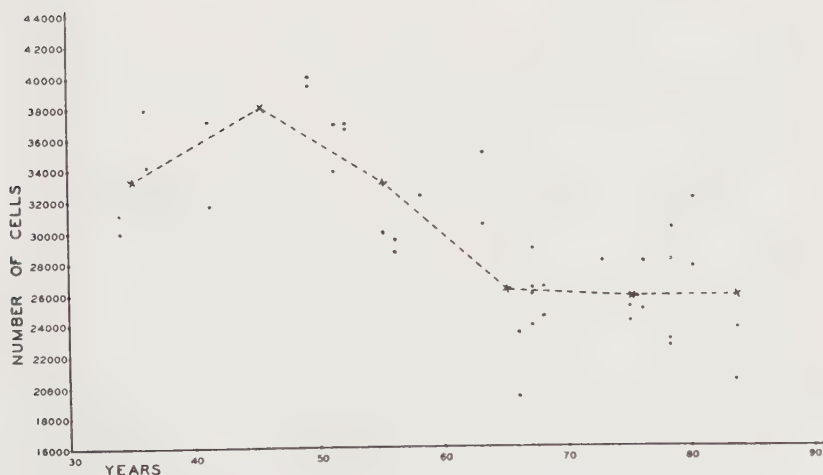


FIG. 3 FIELD GRAPH OF THE NUMBER OF GANGLION CELLS IN 8TH AND 9TH THORACIC DORSAL ROOT GANGLIA OF 21 BODIES

fifth decade, followed by a sharp decline to the seventh decade, and a leveling off. The decade averages for each ganglion, and the averages for both ganglia in each body, are given in table 3. The two cases in the fifth decade gave a count 13% higher than the two in the fourth decade. The five cases from the sixth show a 11.5% decrease from those of the fifth decade. The five from the seventh decade show a decrease of 30.3%; the five from the eighth a decrease of 30.4%; and the two from the ninth decade a decrease of 30.3%.

It is of interest that the ganglion cells exhibit other signs of senility than a decrease in number. Ganglion cells often are

TABLE 3
Averages by decades of ganglion cell counts in twenty-one bodies

	30-39	40-49	50-59	60-69	70-79	80-89
8T DRG	32832	39281	34269	25868	26043	24367
9T DRG	34115	36367	32695	27062	26592	28348
Averages of 8T and 9T DRG	33473	37828	33482	26365	26317	26357
Per cent of increase over the 30-39 year group		13%				
Per cent of decrease from the 40-49 year group			11.5%	30.3%	30.4%	30.3%

pigmented early in life. With increasing age this pigment not only becomes more abundant but is found in many more cells. It may be yellow or dark brown in color, the darker being composed of finer granules, more closely packed together. Kunz ('29) likewise found that autonomic ganglion cells contain two kinds of pigment; a yellow lipid, and a darker melanotic which may be intimately mixed. Both are present in spinal ganglion cells and are related to age. The pigment usually is located on one side of the nucleus, forming an irregular or crescent-shaped mass, but may completely encircle it. In older ganglia in which excessive pigmentation is common, the pigment is usually of the melanotic type and occasionally may be so abundant throughout the cell as to obscure the nucleus.

Another characteristic of the older ganglia is the presence of the "frayed cells" of Cajal ('28). These are atypical cells, indicative of neuron decay due to age or degenerative lesions. They show frayed and festooned outlines and are deeply indented by numerous satellite cells. The interstitial connective tissue is usually increased in amount in older ganglia, the capillary bed appears to be increased, and numbers of dilated blood vessels may be present. Occasionally one also sees nuclei in the process of extrusion.

DISCUSSION

These root fiber counts confirm the previous ones and indicate a marked decrease in the number of myelinated fibers in human spinal roots with age. The 12.7% decrease calculated from the present series represents only the decrease from the fourth decade. Since counts on younger bodies were not made, the present were combined with the previous counts giving a total of sixty-one cases. This is twice as many as before. The largest age group, 70-79 inclusive, which contained sixteen bodies, showed the greatest decrease in the number of myelinated fibers, 27%, and the 60-69 group of thirteen bodies revealed a 24.7% reduction. These two groups probably show the decrease more accurately than the 80-89 group, which contained only five cases, and revealed a reduction of 21.6%. Although the unmyelinated fibers were not counted, they undoubtedly undergo a comparable decrease for the counts indicate that the loss of ganglion cells is several times that of myelinated dorsal root fibers.

Davenport and Bothe ('34) counted 30,500 cells in a ninth thoracic spinal ganglion, 10,500 myelinated fibers in the dorsal root, and 5,600 myelinated fibers in the ventral root of a 45-year-old body. These counts agree quite well with those of this series for the same age. The decrease in the number of ganglion cells must result in a decrease in the number of dorsal root fibers, and indicates that the decrease in the counts of the myelinated fibers was not an error occasioned by a loss of myelin alone. The reduction in the counts of ventral root

these suggest that this loss is due to degeneration of anterior horn cells, which could be established by a similar procedure. The only conclusion that can be reached from the present series of ganglion cell counts is that after the fifth decade there is a marked decrease in the number of cells, amounting to almost one-third. The increase in number of cells from the fourth to the fifth decade is probably an apparent increase occasioned by an insufficient number of ages in the earlier decades. Since the decrease in number of dorsal root fibers begins after the third decade, counts of spinal ganglion cells must be made in series of these ages to determine whether or not reduction in the number of spinal ganglion cells actually begins at the same time.

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